PHD

# The synthesis of myo-inositol phosphate analogues which interact with the receptors and enzymes of the ins(1,4,5)P3 signalling pathway

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# THE SYNTHESIS OF *MYO*-INOSITOL PHOSPHATE ANALOGUES WHICH INTERACT WITH THE RECEPTORS AND ENZYMES OF THE INS(1,4,5)$P_3$ SIGNALLING PATHWAY

A Thesis submitted by Stephen Joseph Mills
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**ABSTRACT**

In this thesis, the synthesis of novel analogues of the second messenger D-*myo*-inositol 1,4,5-trisphosphate is described. The first four sections of the thesis describe the biology and chemistry of the analogues of *myo*-inositol 1,4,5-trisphosphate.

DL-3,6-Di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol was treated with acid to give DL-1,4-di-*O*-benzoyl-*myo*-inositol. Phosphitylation of this compound with diethoxychlorophosphine followed by oxidation with *t*-butylhydroperoxide gave the fully protected, phosphorylated compound. The eight ethyl groups were removed with bromotrimethylsilane and basic hydrolysis of the intermediate gave DL-*myo*-inositol 1,2,4,5-tetrakisphosphate.

Treatment of DL-1,4-di-*O*-benzyl-5,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol with *S*-(+)-*O*-acetylmandelic acid and dicyclohexylcarbodiimide acylated the 3-position selectively to give two diastereoisomers which were separated by flash chromatography. The acyl groups of the individual diastereoisomers were hydrolysed followed by acidic hydrolysis of the *p*-methoxybenzyl ethers to give D- and L-3,6-di-*O*-benzyl-*myo*-inositol. Phosphitylation of the tetrols using bis(benzyloxy)diisopropylaminophosphine and 1*H*-tetrazole followed by oxidation with *m*CPBA gave the protected 1,2,4,5-tetrakisphosphate. Alternatively, sulphoxidation of the D- enantiomer with sulphur in pyridine-DMF gave the protected 1,2,4,5-tetrakisphosphorothioate. Deprotection of the individual compounds with sodium in liquid ammonia gave D- and L-*myo*-inositol 1,2,4,5-tetrakisphosphate and D-*myo*-inositol 1,2,4,5-tetrakisphosphorothioate.

DL-1,4-Di-*O*-allyl-*myo*-inositol was *p*-methoxybenzylated selectively to give DL-1,4-di-*O*-allyl-3,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol. This intermediate was methylated at the 2- and 5-positions followed by deprotection of the allyl and *p*-methoxybenzyl groups to give 2,5-di-*O*-methyl-*myo*-inositol. 2,5-Di-*O*-benzyl-*myo*-inositol was synthesised in a similar way. Each of these tetrols were phosphitylated and the 2,5-di-*O*-methyl-*myo*-inositol derivative was oxidised or sulphoxidised followed by deprotection of the phosphate triester whereas the 2,5-di-*O*-benzyl-*myo*-inositol derivative was sulphoxidised and deprotected to give *myo*-inositol 1,3,4,6-tetrakisphosphorothioate.

DL-1,2,5-Tri-*O*-benzyl-*myo*-inositol was prepared in several steps from DL-3,6-di-*O*-allyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol. This triol was phosphitylated and oxidised or sulphoxidised followed by deprotection with sodium in liquid ammonia to give DL-1,4,6-*myo*-inositol trisphosphate and DL-*myo*-inositol 1,4,6-trisphosphorothioate respectively.

DL-1,4-Di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol was selectively acylated at the 3-position using *S*-(+)-*O*-acetylmandelic acid and dicyclohexylcarbodiimide to give two diastereoisomers which were separated by flash chromatography. Basic hydrolysis of the ester group followed by several protection and deprotection steps gave D- and L-2,3,5-tri-*O*-benzyl-*myo*-inositol. Phosphitylation and oxidation followed by deprotection of both isomers gave the chiral 1,4,6-trisphosphates. Sulphoxidation and deprotection of the D- isomer gave D-*myo*-inositol 1,4,6-trisphosphorothioate.

Phosphitylation of benzene-1,2,4-triol with diethoxychlorophosphine followed by ethyl group deprotection using bromotrimethylsilane gave benzene-1,2,4-trisphosphate.

## Dedication

This thesis is dedicated to my parents, Allen and Josephine Mills, my brother Tony, and sisters Catherine, Helen, Elizabeth and my nephew David, who have given me constant encouragement and love throughout the years of research.

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## Publications

Part of the work described herein has appeared in the following publications:

Stephen J. Mills, Stephen T. Safrany, Robert A. Wilcox, Stefan R. Nahorski and Barry V. L. Potter, "Synthesis of *myo*-Inositol 1,2,4,5-Tetrakisphosphate, A $Ca^{2+}$-Mobilising Tetrakisphosphate With A Potency Similar To *myo*-Inositol 1,4,5-Trisphosphate", *Bioorg. Med. Chem. Lett.,* 1993, **3**, 1505–1510.

Stephen J. Mills, Jenan Al-Hafidh, John Westwick and Barry V. L. Potter, "*myo*-Inositol 1,4,6-Trisphosphate: A New Synthetic $Ca^{2+}$-Mobilising Inositol Phosphate", *Bioorg. Med. Chem. Lett.,* 1993, **3**, 2599–2604.

Robert A. Wilcox, Stephen T. Safrany, Dethard Lampe, Stephen J. Mills, Stefan R. Nahorski and Barry V. L. Potter, "Modification at C2 of *myo*-inositol 1,4,5-trisphosphate produces inositol trisphosphates and tetrakisphosphates with potent biological activities", *Eur. J. Biochem.,* 1994, **223**, 115–124.

Stephen T. Safrany, Stephen J. Mills, Changsheng Liu, Dethard Lampe, Nicholas J. Noble, Stefan R. Nahorski and Barry V. L. Potter, "Design of Potent and Selective Inhibitors of *myo*-inositol 1,4,5-Trisphosphate 5-Phosphatase", *Biochemistry,* 1994, **33**, 10763–10769.

**Abbreviations**

| | |
|---|---|
| All | allyl |
| ADP | adenosine 5′-diphosphate |
| AIBN | *azo-bis*-isobutyronitrile |
| AMP | adenosine 5′-monophosphate |
| ATP | adenosine 5′-triphosphate |
| Bn | benzyl |
| Bz | benzoyl |
| cAMP | adenosine 3′,5′-cyclic monophosphate |
| °C | degrees celcius |
| CAN | ceric ammonium nitrate |
| CICR | calcium induced calcium release |
| COSY | correlated spectroscopy |
| *m*CPBA | *meta*-chloroperbenzoic acid |
| CRBHS | crude rat brain homogenate supernatant |
| DABCO | 1,4 diazabicyclo[2.2.2]octane |
| DAG | 1,2-diacylglycerol |
| DAST | diethylaminosulphur trifluoride |
| DCC | dicyclohexylcarbodiimide |
| DDQ | 2,6-dichloro-3, 5-dicyano-*p*-benzoquinone |
| DMAP | *N,N*-4-dimethylaminopyridine |
| DMSO | dimethylsulphoxide |
| DMF | *N,N*-dimethylformamide |
| $EC_{50}$ | concentration of agent producing 50% of maximal response |
| EE | 1-ethoxyethyl |
| Et | ethyl |
| G-protein | GTP-binding protein |
| FAB | fast atom bombardment |
| GTP | guanosine 5′-triphosphate |
| h | hour |
| HEG | human erythrocyte ghost |
| HMPA | hexamethylphosphoric triamide |
| HPLC | high performance liquid chromatography |
| $IC_{50}$ | concentration of compound inhibiting a response by 50% |
| $Ins(1,4,5)P_3$ | *myo*-inositol 1,4,5-trisphosphate |

| | |
|---|---|
| $Ins(1,3,4,5)P_3$ | *myo*-inositol 1,3,4,5-tetrakisphosphate |
| $Ins(1,4,5)PS_3$ | *myo*-inositol 1,4,5-trisphosphorothioate |
| kDa | kilodaltons |
| $K_i$ | dissociation constant for an inhibitor-enzyme complex |
| $K_m$ | michaelis-menten constant |
| Me | methyl |
| MEM | 2-methoxyethoxymethyl |
| μM | micromolar |
| mM | millimolar |
| min | minute(s) |
| NMR | nuclear magnetic resonance |
| Ph | phenyl |
| PLC | phospholipase C |
| PMB | *p*-methoxybenzyl |
| PKC | protein kinase C |
| Prop | prop-1-enyl |
| PtdIns | phosphatidylinositol |
| $PtdIns(4,5)P_2$ | phosphatidylinositol 4,5-bisphosphate |
| RBL | rat basophilic leukemic |
| TEAB | triethylammonium bicarbonate |
| THF | tetrahydrofuran |
| TIPS | 1,1,3,3-tetraisopropyldisiloxane |
| TLC | thin layer chromatography |
| UV | ultra-violet |
| $v_{max}$ | maximum velocity of an enzyme-catalysed reaction |

CONTENTS

**CHAPTER THREE: A Review Of *myo*-Inositol Phosphate Synthesis**

**CHAPTER SIX: Experimental**

# CHAPTER ONE

# A Brief History Of Second Messengers

## 1.1 General Introduction to Communication Between cells

The cell receives a plethora of external information from its surrounding environment, which must somehow be translated into the cell to produce an appropriate response. When cells become organised to form an organism, the individual cells need to assess both the general status of the organism in relation to its environment, and the specific functional status of other cells. The individual cells of a multicellular organism need to communicate with each other so that the basic processes of life may occur, for example, growth, movement and metabolic activity. This communication may occur in several ways. First, *via* cytoplasmic bridges or gap junctions, which form a pore between two cells whose plasma membranes are in direct contact, and which facilitate the direct exchange of cytoplasmic contents (for example, some metabolites and certain ions). This type of direct exchange suffers from a slow flow-rate of information between cells. [1,2]

Second, by secreting chemical messengers: these chemical messengers, or first messengers, may be classified into three functional types: (a) hormones, (b) neurotransmitters and (c) local mediators. First, hormones are secreted by endocrine glands, and transported long distances to their respective target organs by the bloodstream. Major endocrine organs include the hypothalamus and pituitary in the brain; thyroid and parathyroid in the neck region, the liver, pancreas, ovaries and testis. There are over two hundred steroid hormones, both natural and synthetic. These are based on the four ring sterane nucleus which is synthesised from cholesterol by cleavage of the aliphatic side chain.

A second class of messenger are the neurotransmitters, which are released by nerve cells called neurones. Sensory neurones for example, take input from the sense organs back to the central nervous system: interneurones, which connect other neurones and perform the complex summation of information which result in the final output, and motor neurones, which take the output from the central nervous system in order to control voluntary muscles. The sympathetic nerve network controls the involuntary contraction of smooth muscle and secretion by endocrine and exocrine glands. The communication between neurones occurs at synapses *via* neurotransmitters. Some neurotransmitters are synthesised *in vivo* e.g. acetylcholine is derived from acetyl co-enzyme A and choline. Some amino acids, for example glycine and glutamate are used as neurotransmitters without modification.

Finally, local mediators form a diverse group of first messengers. Nerve growth factor (NGF) seems to direct the growth of the axon of a sympathetic nerve to its target cell. Platelet-derived growth factor (PDGF) stimulates proliferation of fibroblasts at the site of injury which is involved in the initiation of wound repair. Local mediators such as the eicosanoids, including prostaglandins, are ubiquitous in mammals, with many functions. Purines, for example adenosine, have physiological effects on the same cells which release them. Retinoic acid is a special type of local mediator, gradients of which control the development of digits of the wing in birds.

First messengers, such as those described above, work by binding to a protein receptor on the ectoplasmic surface of the cell, (with the exceptions of steroid and thyroid hormones and retinoic acid), which is then transmitted into a chemical change on the cytoplasmic surface of the membrane. Signal transduction at the receptor protein in the cell works by one of three mechanisms. The first of these is by a receptor, which contains or is closely linked to an ion channel which spans the membrane. For example, the nicotinic acetylcholine receptor has an excitatory cation channel, however, the cell also has two receptor proteins for inhibiting transmitters [$\gamma$-aminobutyrate (GABA) and glycine] that are anion channels and related to the nicotinic receptor. The ion channel receptor of nicotinic acetylcholine has five closely related subunits that associate in a pseudosymmetrical manner and line a central ion channel.

Second, signal transduction may be carried out by second messenger systems. The classical cyclic-3',5'-adenosine monophosphate (cyclic AMP) produces a response to $\beta$-adrenergic agonists and many other first messenger agonists. The second messenger is produced catalytically in response to an agonist or first messenger and the signal is amplified, because it is not produced stoichiometrically. The first messenger in this type of system increases the concentration of the second messenger on the cytoplasmic side of the cell membrane and also mimics the effect of the first messenger.

Adenylate cyclase, the enzyme responsible for producing cyclic AMP, is coupled indirectly to the receptor *via* GTP-binding proteins (G-proteins). The receptors are members of the "seven-pass" family containing seven transmembrane domains. The system outlined above shows analogies to the system involved in light absorbtion by rhodopsin with the activation of cyclic guanosine monophosphate phosphodiesterase in the eye. Several receptors inhibit adenylate cyclase *via* inhibitory G-proteins, which may also be involved in coupling to ion channels.

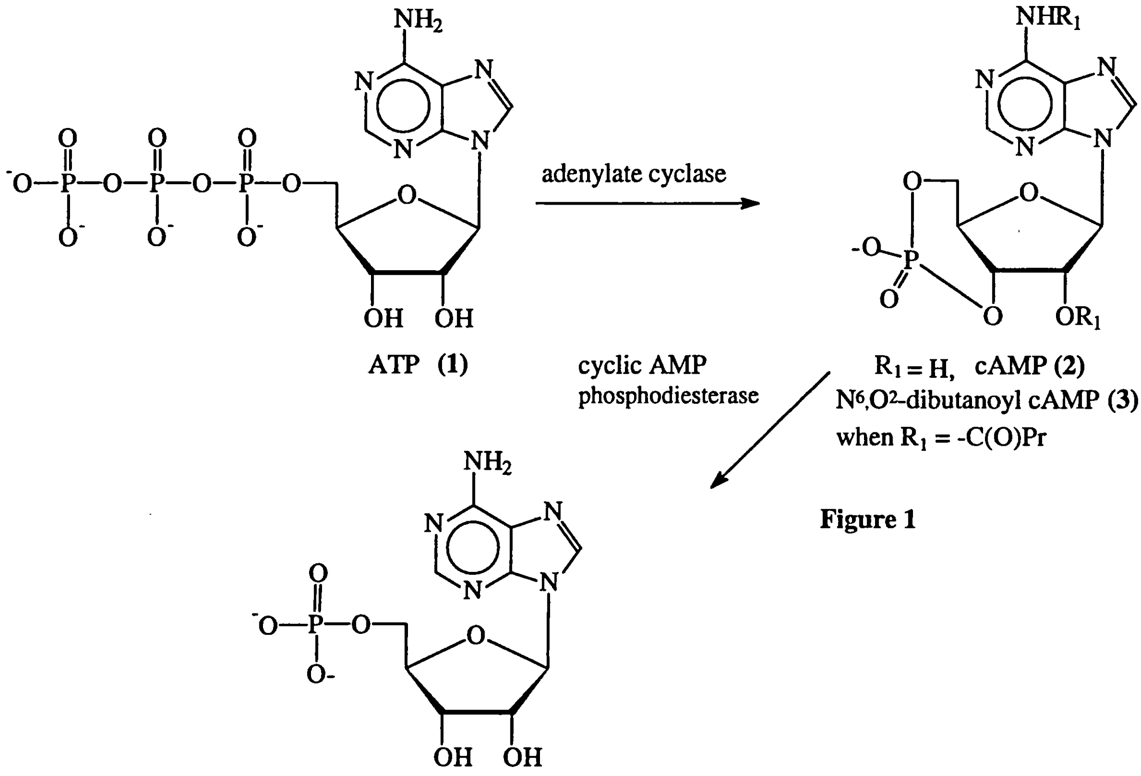
Many agonists that act as first messengers cause an increase in calcium concentration in the cytosol, by releasing the divalent cation from the endoplasmic reticulum. The second messenger in this case is D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)$P_3$], which is released from the phospholipid, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)$P_2$], by the action of phospholipase C (PLC). This enzyme exists in several isoforms, some are coupled to receptors *via* G-proteins. Diacylglycerol (DAG) is another second messenger that is released as a consequence of PtdIns(4,5)$P_2$ hydrolysis, which activates another enzyme, protein kinase C (PKC).

Finally, receptors that contain an integral enzyme activity include the insulin and growth factor class, which exhibit ligand activated protein (tyrosine) kinase activity, and the resact and atrial natriuretic peptide receptors, which directly produce the second messenger, cyclic GMP. [1,2]

**The Discovery of cyclic AMP, cyclic GMP, Ins(1,4,5)$P_3$ and Diacylglycerol**

## 1.2 Cyclic AMP

In the 1950s Sutherland and his colleagues [3,4] discovered cyclic AMP. It was known that adrenaline and glucagon caused the breakdown of glycogen by the increased activity of glycogen phosphorylase in the liver. Cori and Cori (1945) [5] had previously isolated two types of glycogen phosphorylase named *a* and *b* from skeletal muscle. Glycogen phosphorylase *b* required AMP as an activator. [5] Sutherland, Rall and coworkers (1956) [6] showed that ATP (**1**) and $Mg^{2+}$ were necessary for the conversion of inactive glycogen phosphorylase *b* into the active type *a*. Rall and coworkers (1957) [7] developed a cell-free system where an increase in phosphorylase activity occurred after the addition of adrenaline or glucagon to liver homogenate, in the presence of ATP and $Mg^{2+}$ ions. The compound that resulted from this research was a heat stable substance that was shown to activate glycogen phosphorylase in dog liver homogenates in the absence of glucagon or adrenaline. Lipkin and coworkers (1959) [8] were investigating the degradation of ATP by aqueous barium hydroxide. One of the degradation products was found to be similar in biological properties to the heat-stable substance responsible for activating phosphorylase. The two groups collaborated and identified the structure as 3',5'-cyclic adenosine monophosphate (Figure 1). At about the same time Sutherland and Rall [4] discovered adenylate cyclase, then another group followed by discovering cyclic AMP phosphodiesterase; [9] cyclic AMP-dependent protein kinase [10] was discovered in later years. Following the discovery of cyclic AMP, the possibility that it may play a role for the activation of different hormones in other cells was investigated.




ATP (1)

$R_1$ = H, cAMP (2)
$N^6,O^2$-dibutanoyl cAMP (3)
when $R_1$ = -C(O)Pr

AMP (4)

**Figure 1**

The groundwork had been established and the next step was to determine how cyclic AMP was involved in response to a first messenger. Two criteria were put forward: first, the first messenger on binding to its receptor, increases the concentration of cyclic AMP inside the cell: second, increasing the second messenger concentration inside the cell should mimic the effect of the first messenger. (Some of the major roles of cyclic AMP are shown in Table 1).

In unstimulated cell types cyclic AMP is present in low concentration ($10^{-6}$ mol $l^{-1}$), so testing the first criteria needed a sensitive assay. Another problem was that cyclic AMP was not cell permeable. Less polar analogues have been synthesised by modification of the $N^6$ and $O^2$ of cyclic AMP to produce dibutyryl cyclic AMP **(3)** that moves across the plasma membrane faster than cyclic AMP **(2)** itself. These derivatives bind to cyclic

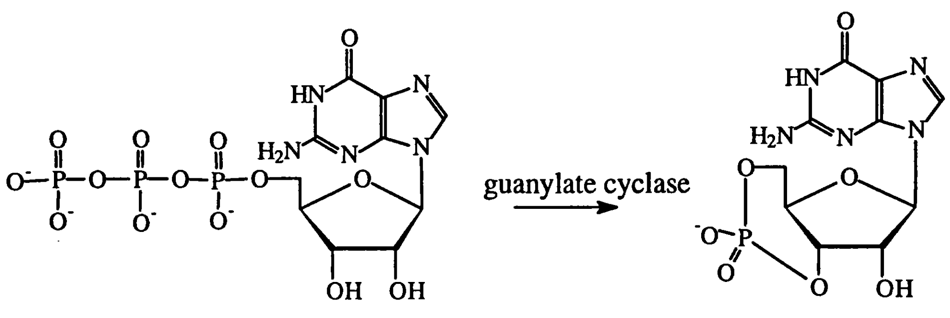
AMP-dependent protein kinase with a similar affinity to cyclic AMP. The cyclic AMP derivative is rapidly metabolised within the cell. The metabolites themselves, may influence cell metabolism and complicate the interpretation of the results from the experiments.

**Table 1** *Some of the roles of cyclic AMP as an intracellular messenger.*

| **Agonist** | **Cell** | **Response** |
|---|---|---|
| Adrenaline (β) | Heart muscle | Modulation of muscle |
| Adrenaline (β) | Smooth muscle | contraction |
| Acetylcholine (M) | | |
| Glucagon ($G_1$) | Liver, kidney, heart muscle | Glucose synthesis and glycogenolysis |
| Glucagon | Pancreatic β cell | Secretion of hormones and |
| Glucose | | proteins |
| Adrenaline | Parotid gland | Secretion of ions and fluid |
| 5-Hydroxytryptamine | Salivary gland | |
| Vasopressin | Kidney | |
| ADP | Blood platelet | Mitogenesis |

### 1.3 Cyclic GMP

3',5'-Cyclic guanosine monophosphate, (**6** in Figure 2) cyclic GMP was discovered by Ashman and coworkers [11] in 1963 which was isolated from mammalian urine. Initally,



GTP (**5**) cyclic GMP (**6**)

**Figure 2**

the detection of cyclic GMP and cyclic GMP-dependent protein kinase, may have suggested that this nucleotide may perform a role similar to that of cyclic AMP. The first description of a function for cyclic GMP was provided by Miki and coworkers [12] and Chader and coworkers [13] who discovered that light activated the cyclic nucleotide phosphodiesterase in photoreceptor cells. It was in 1984 when definitive evidence for an intracellular messenger function for cyclic GMP other than the photoreceptor response was reported. [14] The initial clues came from observations that certain drugs that were used to lower blood pressure (for example nitroprusside) activated the soluble guanylate cyclase in atrial muscle which caused relaxation of the muscle. Recently, it has been found that atrial natriuretic peptides, released by the atria of the heart in response to high blood pressure activate particulate guanylate cyclase in artrial smooth muscle, causing relaxation of the blood vessel wall. The particulate guanylate cyclase that was activated is an integral component of the atrial natriuretic peptide receptor. [14] A closely related system is involved in the response of sea urchin spermatozoa to the chemotactic peptide, resact, released by eggs. It is likely that cyclic GMP has a more widespread role as an intracellular messenger than present knowledge indicates. Experimental difficulties may have prevented the elucidation of roles for cyclic GMP in many cell types (Table 2).

**Table 2** *Some of the roles of cyclic GMP as an intracellular messenger.*

| **Agonist** | **Cell** | **Response** |
|---|---|---|
| Light | Rod and cone cells of the retina | Light-induced nerve impulse |
| Atrial natriuretic peptide | Kidney | Excretion of $Na^+$ and water |
| Atrial natriuretic peptide | Smooth muscle | Muscle relaxation |
| 5-Hydroxytryptamine | Nerve cells | Increased $Ca^{2+}$ inflow |
| Speract and other sperm-activating peptides secreted by egg cells | Spermatazoa | Modification of function of spermatazoa |
| Endothelial-derived relaxing factor (EDRF) | Smooth muscle | Muscle relaxation |

### 1.4 Ins(1,4,5)$P_3$ and Diacylglycerol

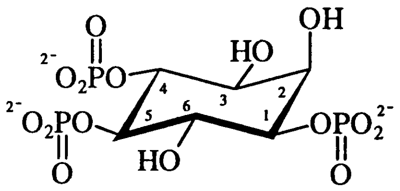
The discovery of the inositol lipid signalling pathway represented a long period of basic research. This was undertaken in two quite unrelated areas which culminated in an understanding, albeit not complete, of a novel mechanism that regulates many cell functions. In 1953 Hokin and Hokin [15] showed that acetylcholine stimulated the incorporation of $[^{32}P]HPO_4^-$ into phospholipids from pancreatic acinar cells. This result was interpreted as the first messenger (acetylcholine) stimulating the synthesis of phospholipid. Further experiments showed that the major phospholipid involved was phosphatidylinositol (PtdIns).

The implications of the experiments performed by Hokin and Hokin [15] were not fully realised until many years later. The reason, in part, was due to many scientists thinking that cyclic AMP and cyclic GMP were the only second messengers in cells. These second messengers were discovered around the same period as Hokin and Hokin initially observed acetylcholine induced-PtdIns turnover. In the period from 1970 to 1975 it was realised that for some agonists, cyclic AMP was not the second messenger which conveys information from the plasma membrane to intracellular sites. Also found, was some indirect evidence which indicated that changes in intracellular calcium concentration were essential for the actions of many agonists. Within the same time period three separate groups (Durell, [16] DeRobertis, [17] and Michell [18]) performed a series of experiments which led to the proposition that agonist-stimulated PtdIns hydrolysis caused an increase in intracellular calcium concentration. [16-19] The key link between phosphoinositides and calcium movement remained undetected for several more years. It was also shown that certain agonists, not only caused an increase in plasma-membrane calcium influx, but also induce the release of calcium from intracellular stores.

The next piece of evidence to suggest a role for phosphoinositides in intracellular signal transduction came from experiments carried out in 1979 by Fain and Berridge. [20] They showed that 5-hydroxytryptamine stimulated calcium movement across the plasma membrane of blowfly salivary glands, which was dramatically decreased by prior removal of the inositol content from the glands. [20] The calcium response was restored by the addition of exogenous *myo*-inositol. The implication of this observation was that inositol metabolites played a crucial role in the action of 5-hydroxytryptamine.

In 1977 Abdel-Latif [21] and his coworkers and Michell and Kirk [22] in 1981 working with iris smooth muscle and liver, respectively, showed that agonists which use calcium as an intracellular messenger induce the rapid hydrolysis of PtdIns(4,5)$P_2$ and phosphatidylinositol 4-phosphate (PtdIns 4P) as well as PtdIns.

The observation that certain agonists stimulate PtdIns(4,5)$P_2$ hydrolysis focused attention on the unique chemistry of the PtdIns(4,5)$P_2$ polar head group. In 1983 Berridge [23] observed that in blowfly salivary gland, 5-hydroxytryptamine increased the concentration of D-*myo*-inositol trisphosphates as well as bis- and monophosphates. In the same year, the critical paper presented in *Nature* by Streb, Berridge, Irvine and Schultz [24] demonstrated the crucial link, in pancreatic acinar cells, between D-*myo*-Inositol 1,4,5-trisphosphate (**7**) [Ins(1,4,5)$P_3$], released as a consequence of PtdIns(4,5)$P_2$ hydrolysis



Ins(1,4,5)$P_3$ (**7**) **Figure 3**

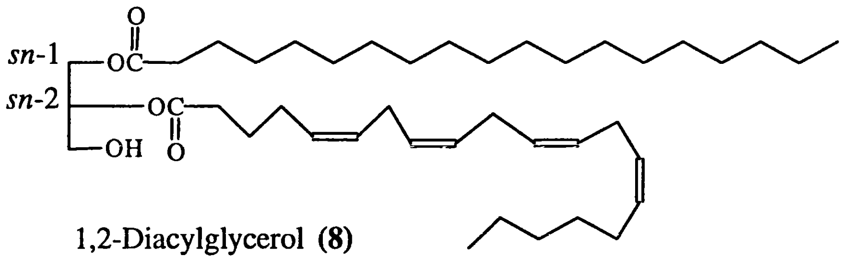
and the release of calcium from intracellular stores. This observation has been repeated in many other cells. (For examples see Table 3).

**Table 3** *Some cellular responses which require the action of Ins(1,4,5)$P_3$ as an intracellular messenger.*

| Cell | Stimulus | Cellular Response |
|---|---|---|
| Nerve cells | Membrane depolarisation | Neurotransmitter release |
| Smooth muscle | Noradrenaline | Muscle contraction |
| Macrophages | Zymogens | Prostaglandin synthesis |
| Unfertilised eggs | Interaction with sperm cell | Cortical granule exocytosis |
| Pancreatic acinar | Cholecystokinin | Amylase secretion |
| Pancreatic β-cells | Glucose | Insulin secretion |
| Photoreceptor | Absorption of light by rhodopsin | Membrane depolarisation |

Meanwhile, Nishizuka [25] and his coworkers discovered a protein kinase that requires calcium and phospholipid for activity, this was named protein kinase C. Nishizuka's group [26,27] observed that diacylglycerols and tumour-promoting phorbol esters are potent activators of protein kinase C. Nishizuka's group proposed that diacylglycerols are formed by the hydrolysis of PtdIns(4,5)$P_2$ and the hydrophobic diacylglycerol (**8** in Figure

4) activates protein kinase C. [26] Some of the roles of diacylglycerol as an intracellular second messenger are given in Table 4




1,2-Diacylglycerol **(8)**

**Figure 4**

**Table 4** *Some intracellular processes which require the action of diacylglycerol as an intracellular messenger.*

| Intracellular Process | Target Proteins for Diacylglycerol-Activated Protein Kinase C |
|---|---|
| Activation of protein-tyrosine kinase receptors | Receptors for EGF, insulin |
| Activation of receptors linked to GTP-binding proteins | β-adrenergic receptors |
| Release of calcium | Voltage operated calcium channels |
| Release of cyclic GMP | Guanylate cyclase |
| Muscle contraction and movement of the cytoskeleton | Myosin light chains (kinase)<br>Cytoskeletal proteins |
| Metabolic pathways | Regulatory enzymes of glycogenolysis and glycolysis. Glucose transport. Tyrosine hydroxylase (neurotransmitter release). |

Figure 5 shows the complex interactions between the signalling systems as depicted by Michell (taken from *TIBS* 1992, **17**, 276).

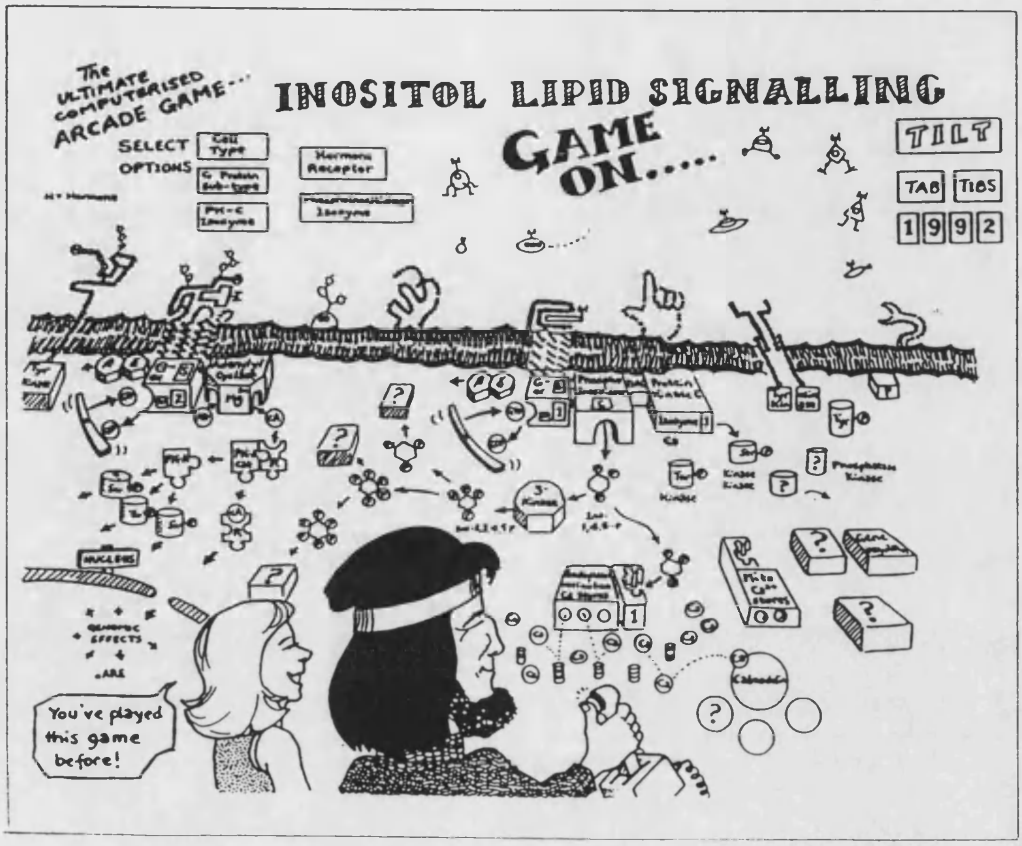



**Figure 5**

# CHAPTER TWO

# A Review Of Second Messenger Pharmacology

## 2.1 Introduction

This chapter will provide a detailed account of the events known to date which link the first messenger (agonist) binding to its receptor, together with the events that follow and the formation and degradation of Ins(1,4,5)$P_3$ and $Ca^{2+}$ release from intracellular stores, and a description of the macromolecules involved in these events. The Ins(1,4,5)$P_3$/DAG pathway operates throughout the life of a cell, starting with gametogenesis, fertilisation, cell proliferation and early development thereafter, continuing through differentiation in order to perform very precise control functions in many plant and animal cells. The overall picture is shown briefly in Figure 6.

## 2.2 G-Protein-linked Receptors

The membrane-spanning transducing unit that is controlled by G-protein-linked receptors has three main parts. First, the receptor that receives the primary messenger; second, the G-proteins, which transduce and amplify the initial signal and third, the enzyme phospholipase C-β, which hydrolyses PtdIns(4,5)$P_2$ to give the hydrophobic DAG and hydrophilic Ins(1,4,5)$P_3$. Tyrosine kinase receptors also generate Ins(1,4,5)$P_3$ and DAG, by interacting with PLC-γ. (See Figure 6).

The primary structure of several plasma-membrane receptors which couple with effector enzymes or ion channels are known in detail, and there are a number of different receptors known to be coupled to phosphoinositide turnover and are given in Table 5. [28] The amino acid sequences of the PtdIns(4,5)$P_2$-linked muscarinic acetylcholine receptor, [29] the adenylate cyclase-linked β-adrenergic receptor [30,31] and the cyclic GMP phosphodiesterase-linked photoreceptor, rhodopsin, [32] were the first to be elucidated. The G-protein-linked receptors discovered so far are composed of a single polypeptide chain having seven membrane-spanning domains connected by extracellular and intracellular loops. These transmembrane domains interact with one another to provide a pocket or pore for the agonist to bind, so inducing the conformation and thus activating the G-protein, which in turn is responsible for activating PLC. The second and third cytoplasmic loops of the receptor have an essential role in activating a member of the G-protein family [33] responsible for stimulating separate members of the PLC family. [34,35]

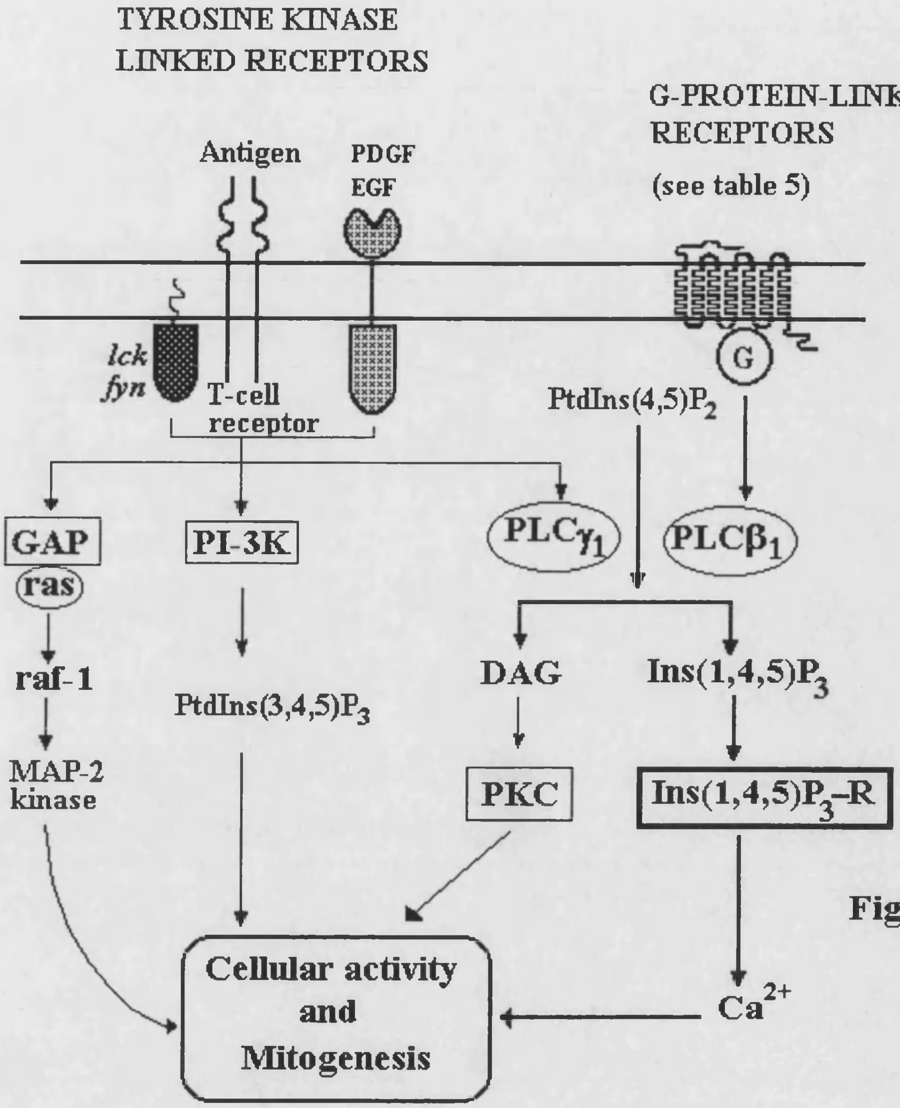



Figure 6

The stereospecific binding site for the first messenger is a sequence of amino acids in the extracellular domain of a G-protein-coupled receptor. For example, by the use of a radioactively labelled agonist which covalently binds to the receptor the precise location of the catecholamine binding site on the β-adrenergic receptor has been found. This methodology has shown that the catacholamine binding site is formed by the juxtaposition of membrane-spanning sequences 3, 4, 5 and 7.

Site-directed mutagenesis in the third loop of the adrenergic-1B receptor resulted in activation of PLC, probably by mimicking the active conformation which is usually induced by the first messenger. [36] Substituting alanine at position 293 (present in loop 3, close to the transmembrane domain 6) with any one of 19 amino acids, resulted in an

**Table 5** *Receptors known to be coupled to phosphoinositide turnover. Compiled from TIPS, 1994 Receptor Nomenclature Supplement.*

| **Receptor Type** | **Endogenous Ligand(s)** |
|---|---|
| $\alpha_1$-Adrenoceptors | Noradrenaline and adrenaline |
| Angiotensin $AT_1$ | Angiotensin II |
| Bombesin $BB_1$ and $BB_2$ | Gastrin releasing peptide, neuromedin B |
| Bradykinin $B_2$ | Bradykinin, kallidin and T-kinin |
| $\alpha$-Chemokine $IL8_A$ $IL8_B$ | Interleukin 8 (IL8), GRO$\alpha$ and (NAP-2) |
| $\beta$-Chemokine MIP1$\alpha$, MCP1 | MIP$\alpha$, RANTES and MCP-1 |
| Cholecystokinin ($CCK_A$, $CCK_B$), gastrin | Cholecystokinin ($CCK_4$, $CCK_8$, $CCK_{33}$,) and gastrin |
| Endothelin $ET_A$ and $ET_B$ | Endothelins 1-3 (ET-1, ET-2, ET-3) |
| Glutamate $mGluR_1$ and $mGluR_5$ | Glutamate |
| Histamine $H_1$ | Histamine |
| $5\text{-}HT_{2A\text{-}2C}$ | 5-HT (hydroxytryptamine) |
| Leukotriene $LTB_4$ and $LTD_4$ | $LTB_4$, $LTC_4$, $LTD_4$, $LTE_4$ |
| Muscarine $M_1$ and $M_3$ | Acetylcholine |
| Neurotensin | Neurotensin and neuromedin N |
| Platelet activating factor (PAF) | PAF and C-PAF |
| Prostanoid $EP_1$ and $EP_3$ | $PGE_2$ |
| FP | $PGF_{2\alpha}$ |
| TP | $TXA_2$ |
| Purinoceptors $P_{2Y}$ and $P_{2U}$ | ATP, ADP, AMP and (UTP $P_{2U}$ only) |
| Tachykinin $NK_{1\text{-}3}$ | Substance P, neurokinins A + B and neuropeptide K and $\gamma$ |
| Vasopressin | Vasopressin |
| Oxytocin | Oxytocin |

**Key:** MIP$\alpha$ = Human macrophage inflammatory protein-1$\alpha$.
RANTES = Human reduced upon activation normal T-expressed and secreted.
MCP-1 = Human monocyte chemoattractant protein-1.
LT = Leukotriene, TX = Thromboxane.

increase in PLC activity. It may be concluded that the native receptor has evolved a loop structure that provides the lowest basal activity. Transfection of cells with mutant receptors showing the highest constitutive activity results in transformation and tumorgenesis. [37]

Site-directed mutagenesis in the third loop of the adrenergic-1B receptor resulted in activation of PLC, probably by mimicking the active conformation which is usually induced by the first messenger. [36] Substituting alanine at position 293 (present in loop 3, close to the transmembrane domain 6) with any one of 19 amino acids, resulted in an increase in PLC activity. It may be concluded that the native receptor has evolved a loop structure that provides the lowest basal activity. Transfection of cells with mutant receptors showing the highest constitutive activity results in transformation and tumorgenesis. [37]

### 2.3 G-Proteins

G-proteins are a family of heterotrimeric guanine nucleotide-binding proteins that serve a critical role in transducing and amplifying signals across the plasma membrane to stimulate PLC. The G-proteins are part of a larger superfamily of GTPases that include factors involved in protein synthesis, for example elongation factor Tu (EF-Tu) and a larger number of monomeric 20-25kDa proteins such as *ras* p21.

#### 2.3.1 Structure and properties

G-proteins consist of three subunits: $\alpha$ (molecular mass = 39-46kDa), $\beta$ (35-37kDa) and $\gamma$ (8kDa). The $\alpha$-subunit possesses the binding site for GTP and the catalytic activity responsible for the hydrolysis of the nucleotide. The $\alpha$-subunit also contains sites which interact with the $\beta\gamma$ complex, with the receptor, and with the effector enzyme or ion channel.

Each of the subunits ( $\alpha$, $\beta$ and $\gamma$) of a G-protein is attached to the cytoplasmic side of the plasma membrane. None of the subunits contain a membrane-spanning sequence. The $\alpha$ -subunit is attached to the membrane through an acylated cysteine residue near the carboxyl terminus of the polypeptide chain. A possible function of the $\beta$- and $\gamma$- subunits, which have both hydrophilic and hydrophobic sequences, may be to assist the anchoring of α-subunits to the plasma membrane.

The functional domains of the α-subunit have been identified and characterised by biochemical, immunochemical and molecular biological approaches. It has been shown that a region of 1-2kDa at the amino-terminal is involved in binding to the βγ-subunit. At the other end of the molecule, the carboxyl terminus is critical for receptor interactions. There is evidence for the latter; for example, a mutation from arginine to proline at the sixth residue from the carboxyl terminus uncouples the G-protein from its receptor. [38] Pertussis toxin ADP-ribosylates those α-subunits which possess a specific cysteine residue near the carboxyl terminus, thereby uncoupling the G-protein from its receptor. Antibodies to the carboxyl terminal decapeptide also uncouple the G-protein from its receptor. [39] Cholera toxin also catalyses the transfer of the ADP-ribose moiety of NAD to a specific arginine residue in certain α-subunits. This modification reduces intrinsic GTPase activity and leads to constitutive activation of the α-subunits. Mutations of this specific arginine residue within the guanine nucleotide-binding region (compare to *ras* position 61) act in the same way to reduce GTPase activity. [38,40]

The α-subunit is in an inactive conformation when GDP is bound. By analogy with the X-ray crystallographic studies of *ras* p21, [41] when GTP binds to the α-subunit, a conformational change is observed which is associated with activation. [38] Several studies have suggested that an aluminium fluoride complex, $AlF_4^-$ activates the α-subunit by mimicking the γ-phosphate of GTP. [42,43] A mutation of a glycine residue comparable to *ras* position 60 prevents the activation of α-subunits by GTP or $AlF_4^-$, presumably by preventing the specific conformational change engendered by the γ-phosphate of GTP.

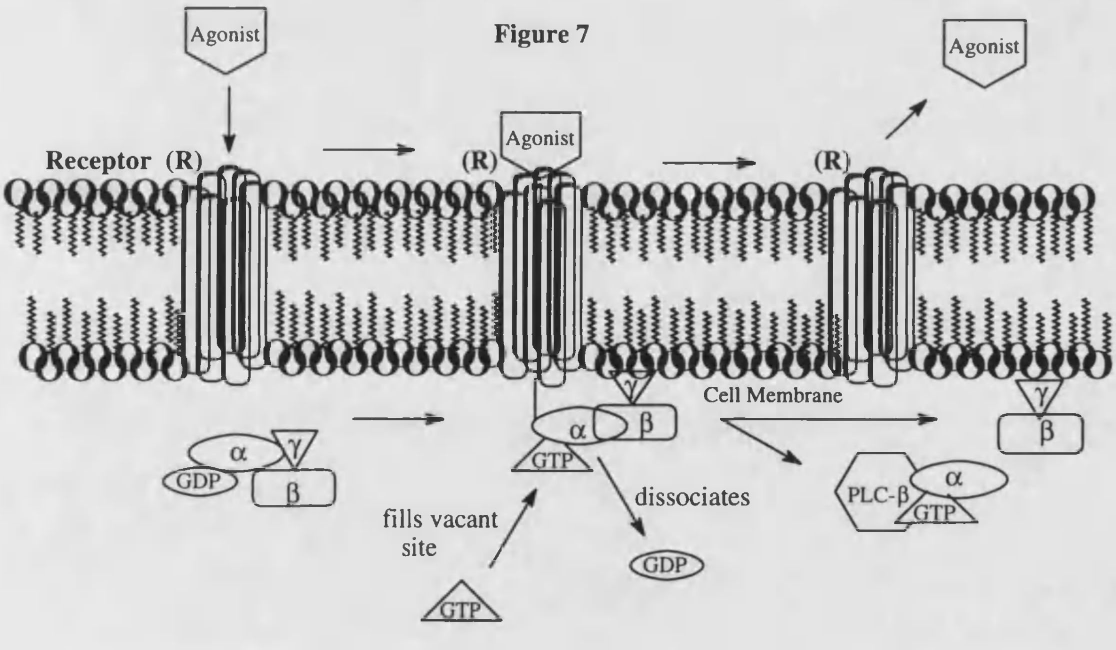
The β-subunits show a repetitive segmented structure made up of contiguous homologous domains, approximately 43 amino acids long and delineated by a tryptophan-aspartic acid motif. [44] Together with the γ-subunit, the βγ-heterodimer serves an essential role in G-protein-receptor coupling. [45] The role of this complex in effector regulation is controversial. The βγ-complex activates an undefined form of PLC in HL-60 granulocytes. [46] The βγ-complex appears to have effects on $K^+$ channel activity, although it is not known if these effects are mediated directly or indirectly. [47] The βγ-complex also plays an obligatory role in agonist-induced receptor phosphorylation and desensitisation. [48]

G-Proteins can be divided into four major subfamilies according to amino acid sequence relationships; these include $G_s$, $G_i$, $G_q$, and $G_{12}$. Recent findings [49-53] have identified the $G_q$ family as regulators of the activation of PLC-β1, which in turn hydrolyses PtdIns(4,5)$P_2$. The $G_q$ family of G-proteins includes $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$. A mixture of $G\alpha_q$ and $G\alpha_{11}$ has been purified from bovine brain [49] and rat liver; [50] a related protein has also been isolated from turkey erythrocytes. [51] Reconstitution of the proteins with purified PLC-β1 [52,53] or turkey PLC [51] results in specific and marked stimulation of the enzyme.

### 2.3.2 GTPase Cycle

The inactive G-protein (Figure 7) exists as a heterotrimer with GDP tightly bound to the α-subunit; under these conditions the agonist receptor (R) is empty and the effector is inactive (for example PLC-β1). When the agonist binds to the receptor (R), the receptor interacts with the heterotrimer complex to induce a conformational change with the dissociation of GDP from the guanine nucleotide-binding site. Under normal conditions GTP fills the site immediately. The GTP binds to the α-subunit to induce a conformational change with two consequences. The G-protein dissociates from the

**Figure 7**




agonist-receptor complex, reducing the affinity of the agonist for the receptor and thus freeing the receptor for an encounter with a neighbouring inactive G-protein. The binding of GTP to the heterotrimer also leads to reduced affinity of α for βγ, and subunit dissociation occurs. The α-GTP complex is now free and is allowed to fulfil its primary role as a regulator of effectors. In some systems, the free βγ-subunit may interact directly with an effector and modulate the activity of the active complex, or it may act independently at a distinct effector. The α-subunits possess an intrinsic GTPase activity and determine the lifetime of the species and the associated physiological response. The α-catalysed hydrolysis of GTP provides GDP in the binding site and causes dissociation and deactivation of the complex. The GTPase activity of the α-subunit is essentially an internal clock acting as an on/off switch. The α-GDP complex has a high affinity for the βγ-dimer, and subsequent reassociation of α-GTP with the βγ-complex returns the system to a basal state.

### 2.4 Phospholipid-specific Phospholipase C

The enzyme responsible for hydrolysing the phospholipid, PtdIns(4,5)$P_2$ is a phosphodiesterase, of the family of phospholipase C. The function of PLC is to hydrolyse the bond between the bridging oxygen and the phosphate. The products are the hydrophobic diacylglycerol and the water-soluble Ins(1,4,5)$P_3$. The generation of these two second messengers set in motion many events that culminates in a particular cellular response, for example growth, movement and metabolism. A large number of receptors have been discovered which use this signalling pathway. The enzymes of the PLC family are regulated by receptors *via* two distinct pathways: these have been unambiguously identified as G-protein-mediated and tyrosine kinase-mediated regulation of the different PLC isoforms. Direct protein isolation and molecular cloning studies have revealed the presence of many PLC isoforms in mammalian tissue. The PLC isoforms are activated by different receptors and different mechanisms and their inhibition is mediated by several mechanisms, for example cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC).

#### 2.4.1 Phospholipase C Isoforms

Classification of PLCs for this review will be based upon sequence homology which includes only those enzymes with known amino acid sequences deduced from their respective complementary DNA (cDNA). Three families of phospholipase C have been established, β, γ and δ. Each family has several subtypes which are designated by adding an arabic numeral after the Greek letter, for example PLC-β1, PLC-γ1. [54]

Although the amino acid sequence homology between different families of PLC is low, there is significant sequence homology shared by the three families, arbitrarily designated X and Y of ~ 170 amino acids and ~ 260 amino acids, respectively. [55] All the PLCs contain an amino-terminal of ~ 300 amino acids that precede the X region. Both PLC-β and PLC-δ contain sequences of 50-70 amino acids which separate the X and Y regions, whereas PLC-γ is characterised by a longer sequence of ~ 400 amino acids, which contain the *src* homology (SH2 and SH3) domains; these were identified as noncatalytic regions common to a variety of *src* family tyrosine kinases. [56] In PLC-β the carboxyl terminal sequence which follows the Y region is ~ 450 amino acids long, but is nonexistent in PLC-δ.

### 2.4.2 Properties of PLC's

All three families of PLC are dependent on calcium. [57-59] The Y sector of the PLC has tentitively been suggested as the binding site for $Ca^{2+}$ on the evidence that this region is homologous to the $Ca^{2+}$-binding domains of PKC and cytosolic phospholipase $A_2$. [56] PLC-β, PLC-γ and PLC-δ hydrolyse all three common *myo*-inositol-containing phospholipids: phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns 4P), phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)$P_2$]. The selectivity for PtdIns(4,5)$P_2$ over PtdIns, decreases in the order PLC-β1 > PLC-γ1 > PLC-δ. The hydrolysis of the three types of *myo*-inositol phospholipid may yield cyclic and noncyclic *myo*-inositol phosphates. [61] It has been demonstrated that cells containing lower than normal concentrations of cyclic *myo*-inositol phosphates grow to a lower density at confluence than cells with normal concentrations. [62] A stereochemical analysis at the chiral phosphorus of the *myo*-inositol phosphate, in the presence of $H_2{}^{18}O$ has shown that PLC initially generates cyclic *myo*-inositol phosphate which is then released or converted into the noncyclic *myo*-inositol phosphate. [63]

### 2.4.3 Activation of the PLC-β family

The nature of the PLC-β remained unknown until recently when the G-protein subfamily $G_q$, was identified and characterised by several laboratories (see section 2.3.1). A number of cDNAs have been sequenced and characterised which correspond to the α-subunits of the $G_q$ subfamily. There are at least five distinct members of this family which have been named (see section 2.3.1). There is considerable homology in amino acid sequence between $G\alpha_q$ and $G\alpha_{11}$ with 88% of the amino acids being identical. However, $G\alpha_{14}$ and $G\alpha_{16}$ possess only 55-60% homology with $G\alpha_q$, and none of the members of the $G_q$ family possesses a site for pertussis toxin modification.

A mixture of $G\alpha_q$ and $G\alpha_{11}$ has been purified [53] which activates PLC from bovine liver membranes that have been incubated with GTPγS. (GDP exchanges very slowly with GTPγS). When $G\alpha_q$ and $G\alpha_{11}$ were reconstituted in the presence of GTPγS, only PLC-β 1 was activated and not PLC-γ1 or PLC-δ1. [53] The affinity of the G-protein mixture for GTPγS was low, being 4μM for half-maximal activation. [64] $AlF_4^-$ also activated PLC-β 1 in the presence of $G\alpha_q$ and $G\alpha_{11}$. The two G-proteins were resolved and both were shown to stimulate the activity of PLC-β1.

The receptors which activate PLC-β *via* the $G_q$ family are given in Table 5. It has been established that the second and third intracellular loops within the $M_1$ muscarinic acetylcholine receptor interact with the PLC-activating G-protein. [65] When the agonist occupies the receptor, the heterotrimeric GDP-bound $G\alpha_q$ dissociates to give the GTP-bound $G\alpha_q$ complex which remains in the membrane. PLC-β1 then binds the GTP-bound $G\alpha_q$ *via* the carboxyl-terminal region of $G\alpha_q$ and $G\alpha_{11}$, which results in the activation of PLC-β1.

The interaction between the Gα-subunits and PLC-β1 have been analysed further by introducing the corresponding cDNA into COS-7 cells and measuring the corresponding formation of *myo*-inositol phosphates, formed after stimulation with aluminium fluoride $AlF_4^-$. [66] Cotransfection of $G\alpha_q$ (or $G\alpha_{11}$) cDNA and PLC-β1 cDNA resulted in even higher levels of *myo*-inositol phosphate formation. A mutation of Gln-209 to Leu which constitutively activates $G\alpha_q$ and $G\alpha_{11}$ resulted in persistent activation of PLC and higher levels of *myo*-inositol phosphates. However, transfection with cDNAs of other G-proteins did not increase *myo*-inositol phosphate formation. Thus, only $G\alpha_q$ and $G\alpha_{11}$ activates PLC-β1 responsible for *myo*-inositol phosphate formation.

Purified $G\alpha_q$ only stimulated PLC-β1 and not PLC-β2. [67] All the G-proteins of the $G_q$ family stimulate PLC-β1, with $G\alpha_q$ and $G\alpha_{11}$ being most effective. G-Protein $G\alpha_{16}$ was found to activate PLC-β2 most effectively, thus there appears to be specificity for the interaction of certain $G_q$-proteins with different members of PLC-β enzymes. This specificity is probably important in generating receptor-specific responses *in vivo*.

The G-protein signalling transduction pathway has been established for the peptide cytokine interleukin-8 (IL-8). [68] Interleukin-8 is one of the most potent chemoattractants for neutrophils. [69] Interleukin-8 also induces angionesis, mediates cytokine-induced transendothelial neutrophil migration, [70] and triggers many other effects associated with the inflammatory response. [69] The complementary DNA (cDNAs) which encodes two distinct types of IL-8 receptors $\alpha$ and $\beta$, has been cloned and sequenced. [71-73] These IL-8 receptors couple to G-proteins and activate PLC in neutrophils. [69] In order to determine whether the two IL-8 receptors, IL-8R$\alpha$ and IL-8R$\beta$, transduce signals *via* the known $G_q$ family, it was necessary to cotransfect COS-7 cells with cDNA's encoding IL-8R$\alpha$ or IL-8R$\beta$ either alone or together with $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, or $G\alpha_{16}$. However, reconstitution was not observed with cells that overexpressed $G\alpha_q$, or $G\alpha_{11}$. The IL-8 receptors were able to interact with endogenous pertussis toxin-sensitive G-proteins or with the recombinant $G_i$-protein, which released free $\beta\gamma$-subunits and activated PLC-$\beta$2. Thus it was found that the cytokine IL-8 acted through G-proteins *via* PLC-$\beta$2 with an increase in *myo*-inositol phosphates. The enzyme PLC-$\beta$2 may therefore be a potential target for the selective action of anti-inflammatory drug design. [68]

### 2.4.4 Regulation of PLC-β Mediated by Protein Kinase C

There is evidence [74] to support the claim that protein kinase C and protein kinase A (in other systems) attenuate receptor-coupled PLC activity in different cell types, which provides a negative feedback loop so as to control precisely the receptor signalling. PLC-$\beta$1 can be phosphorylated by protein kinase C, but is not a substrate for protein kinase A. Phorbol myristate acetate (PMA) stimulated the phosphorylation of a serine residues (Ser-887) of PLC-$\beta$1, but not of PLC-$\delta$1, and only a small increase for PLC-$\gamma$1 in cell lines PC12, C6BuI and NIH 3T3 was observed. [74] Protein kinase C can also phosphorylate stoichiometrically Ser-887 of PLC-$\beta$1 *in vivo*. It has been proposed that the phosphorylation of PLC-$\beta$1 by protein kinase C may alter its interaction with $G_q$. [74]

### 2.5 Types of *myo*-Inositol Phospholipids

There are several types of *myo*-inositol containing phospholipids: these include phosphatidylinositol (PtdIns) **(9)**, phosphatidylinositol 4-phosphate [PtdIns(4)P] **(10)**, and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)$P_2$] **(11)** (Figure 8) which together constitute 2-8% of the total phospholipid. PtdIns accounts for over 80% of the total *myo*-

inositol phospholipid [75,76] and is mainly found in the endoplasmic reticulum: the distribution of other phospholipids is however unknown.

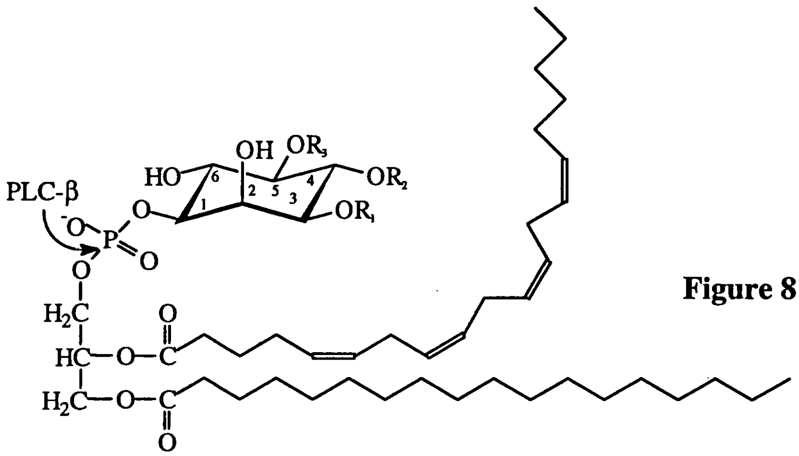


**Figure 8**

(9) $R_1 = R_2 = R_3 = H$; PtdIns

(10) $R_2 = PO_3^{2-}, R_1 = R_3 = H$; PtdIns(4)P

(11) $R_2 = R_3 = PO_3^{2-}, R_1 = H$; $PtdIns(4,5)P_2$

(12) $R_2 = R_3 = H,\ R_3 = PO_3^{2-}$; PtdIns(3)P

(13) $R_3 = H, R_1 = R_2 = PO_3^{2-}$; $PtdIns(3,4)P_2$

(14) $R_1 = R_2 = R_3 = PO_3^{2-}$ ; $PtdIns(3,4,5)P_3$

Recent developments have indicated the presence of several other minor *myo*-inositol phospholipids. Phosphatidylinositol 3-phosphate [PtdIns(3)P] **(12)** has been discovered in intact polyoma middle T-antigen transformed fibroblasts. [77] Later, this phospholipid was also found in non-transformed fibroblasts, [78] astrocytoma cells [79] and in NG 115-401L-C3 neuroblastoma cells. [80] Phosphatidylinositol 3,4-bisphosphate [$PtdIns(3,4)P_2$] **(13)** has been detected in smooth muscle cells. [81,82] Phosphatidylinositol 3,4,5-trisphosphate [$PtdIns(3,4,5)P_3$] **(14)** has also been found in stimulated neutrophils, [83,84] smooth muscle cells, macrophage cell lines and rat cerebrum. [81,85,86] The identification of the *myo*-inositol phospholipids was based upon deacylation and degradation of these lipids, followed by ion exchange high performance liquid chromatography of the remaining *myo*-inositol phosphate moiety. The level of PtdIns(3)P, $PtdIns(3,4)P_2$ and $PtdIns(3,4,5)P_3$, in macrophage cell lines, is 0.2-3.2% of the levels of PtdIns(4)P. Furthermore, these *myo*-inositol phospholipids are present in trace amounts and they are not substrates for phospholipase C. [78] The 3-phosphoinositides may possibly undergo further metabolism, for example dephosphorylation. [87]

### 2.5.1 Phosphoinositide Kinases

Recently, distinct forms of phosphoinositide kinase have been isolated and characterised. Phosphoinositide 3-kinase was purified from fibroblasts [88] and later, to near homogeneity from bovine brain cytosol. [89] Phosphoinositide 3-kinase specifically phosphorylates PtdIns at the 3-position, has a high affinity for ATP and is strongly inhibited by detergents. Phosphoinositide 3-kinase will also phosphorylate PtdIns(4)P and PtdIns(4,5)$P_2$ to give the respective products PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$. [89] Another phosphoinositide kinase with a lower molecular mass than phosphoinositide 3-kinase, may be distinguished from the latter enzyme in that it is an integral membrane protein that only phosphorylates the 4-position of PtdIns, and is activated by detergents and inhibited by adenosine. A third type of phosphoinositide kinase present in brain also phosphorylates the 4-position, but has a larger molecular mass and a lower affinity for ATP than the second type of phosphoinositide kinase. Phosphoinositide 5-kinases exist in both soluble and particulate forms. The 5-kinase enzyme phosphorylates PtdIns(4)P but not PtdIns. It was suggested that PtdIns(4)P 5-kinase in brain membranes may be activated by GTP and its nonhydrolysable analogues, an effect apparently not mediated *via* an inhibition of phosphomonoesterase or PLC activities. [90,91] Another way of regulating PtdIns(4)P 5-kinase may be *via* a brain-specific protein $B_{50}$ which upon phosphorylation by protein kinase C inhibits PtdIns(4)P 5-kinase. [92]

### 2.5.2 Phosphatidylinositol 3-Kinase

The enzyme is a heterodimer whose subunit molecular weights are 85kDa and 110kDa. Two complementary DNA clones for the 85kDa subunit p85α and p85β have been isolated. [93,94] The two polypeptides do not possess intrinsic phosphoinositide 3-kinase activity, however, p85α exists in a tightly bound complex with the 110kDa protein, thought to be the catalytic subunit. [95] The p85α and p85β polypeptides possess an amino-terminal SH3 domain and two SH2 domains. The SH2 domains of p85α and p85β allow interaction with phosphotyrosine-containing sequences in activated growth factor receptors and possibly with tyrosine phosphorylated substrates of receptor tyrosine kinases. [96-99] The p85 proteins may serve as adaptors that bring the catalytic domains of the 3-kinase 110kDa subunit into apposition with the activated tyrosine kinases. This association may activate the 3-kinase by translocating it to the inner surface of the plasma membrane where PtdIns(4,5)$P_2$ is located. The translocation of phosphoinositide 3-kinase may be carried out by the SH3 domain of p85 [100] because SH3 domains of other proteins mediate interactions with the cytoskeleton. The larger subunit (p110kDa) also shows sequence homology with VPS34, which has an undefined role in sorting proteins destined for the yeast vacuole, an intracellular hydrolytic compartment. [101] The yeast mutants defficient in VSP34 exhibit decreased phosphoinositide 3-kinase activity. [101]

The evidence that phosphoinositide 3-kinase activation is crucial to mitogenic signalling by growth factor receptor kinases has been gathered from experiments involving the deletion of "kinase insert" regions from the intracellular domains of the platelet-derived growth factor (PDGF) receptor. Deletion of the kinase insert region abolishes the effects of stimulation of the receptors and selectively destroys the ability of the receptor to activate phosphoinositide 3-kinase. [102] Several recent reviews have covered the structure and function of this enzyme. [103,104]

### 2.5.3 Properties and Signalling Role of PtdIns(3,4,5)$P_3$

The occurrence of PtdIns(3,4,5)$P_3$ is well established, however the physiological significance of PtdIns(3,4,5)$P_3$ is not known at present, although it is commonly accepted to be a signalling molecule. PtdIns(3,4,5)$P_3$ is probably not involved in the synthesis of Ins(1,3,4,5)$P_4$ because it is not a substrate for PLC. [78]

Many agonists such as as formylated Met-Leu-Phe (fMLP), platelet-activating factor (PAF), ATP and several nonhydrolysable guanine nucleotides stimulated the accumulation of PtdIns(3,4,5)$P_3$ in intact neutrophils. [105] This accumulation of PtdIns(3,4,5)$P_3$ is rapid and apparently not driven by activation of other known signalling pathways. [106] These factors have led to the suggestion that PtdIns(3,4,5)$P_3$ was a new "second messenger". This proposal is supported by two pieces of evidence. First, the agonists which cause the rapid accumulation of PtdIns(3,4,5)$P_3$ also provide the signal for phosphoinositide 3-kinase activity, which can synthesise PtdIns(3,4,5)$P_3$ *in vitro.* [107] Second, in whole cells PtdIns(3,4,5)$P_3$ may be synthesised by phosphorylation of PtdIns(4,5)$P_2$ at the 3-hydroxyl position. [108] This observation implies that PtdIns(4,5)$P_2$ is being phosphorylated by phosphoinositide 3-kinase. PtdIns(3,4,5)$P_3$ may also be synthesised from PtdIns(3,4,)$P_2$ *via* a phosphoinositide 5-kinase, [109] however, rapid degradation of PtdIns(3,4,5)$P_3$ raises the possibility that an agonist-sensitive lipid phosphatase may drive the accumulation of PtdIns(3,4,5)$P_3$. [108]

The accumulation of PtdIns(3,4,5)$P_3$ is elicited by a collection of receptors found on neutrophils, thought to generate their effects *via* G-proteins. [110] These receptors are unusual, since the majority of them are known to utilise either receptor or *src*-type protein tyrosine kinase activities to transduce their signals. [107] Many protein tyrosine kinase receptors have been shown to cause translocation of phosphoinositide 3-kinase into signalling complexes in a manner that is dependent on both protein tyrosine kinase activity and the ability of the associating phosphoinositide 3-kinase to specifically recognise tyrosine phosphatase targets in the receptor-activated complex. [107] Thus, a direct pathway by which receptors regulating protein tyrosine kinase activities might control a phosphoinositide 3-kinase has been established. No other form of receptor-controlled regulatory mechanism has been shown to interact with phosphoinositide 3-kinase activity. These factors have led to the expectation that the apparently anomalous situation presented by the capacity of G-protein-linked receptors to regulate PtdIns(3,4,5)$P_3$ accumulation can be explained by a G-protein dependent regulation of a protein tyrosine kinase activity. Thus, a number of G-protein-linked receptors have been shown to activate tyrosine phosphorylation of proteins, [111] and cause an increase in phosphoinositide 3-kinase activity in antiphosphotyrosine and/or anti *src*-type protein tyrosine kinase antibody-directed immunoprecipitates. [112] However, in neutrophils these pathways may drive only a small proportion of the rapid accumulation of PtdIns(3,4,5)$P_3$ which may be elicited *via* G-protein-linked receptors and the majority (> 90%) is a result of a pathway which regulates a PtdIns(4,5)$P_2$-directed phosphoinositide 3-kinase activity by a currently undefined mechanism. [112,113] This major pathway for the synthesis of PtdIns(3,4,5)$P_3$ is staurosporine-resistant which accumulates during the first 10-20s in the presence of a maximal effective dose of the agonist fMLP. [112] Thus the predominant staurosporine-resistant pathways for the activation of phosphoinositide 3-kinase in neutrophils are unlikely to contain multiple steps between their G-proteins and the responsive phosphoinositide 3-kinase and could be direct.

It has been found that PtdIns(3,4,5)$P_3$ is a potent activator of protein kinase C in the presence of phosphatidylserine and calcium ions. PtdIns(3,4,5)$P_3$ may also play a role as an intracellular signal molecule which can aid the cross talk between two or more signals, for example the interaction between serine/threonine kinase and threonine/tyrosine kinase. [114] The high negative charge density of PtdIns(3,4,5)$P_3$ also indicates that the lipid may partake in a pH maintenance system, such as the $Na^+/H^+$ antiport mechanism.

### 2.5.4 Other Functions of Phosphoinositides

*myo*-Inositol lipids play a crucial role in the maintenance of the cytoskeleton which is based upon the ability of PtdIns(4,5)$P_2$ to bind to actin-binding proteins, for example gelsolin. The bound PtdIns(4,5)$P_2$ aids the release of gelsolin from the actin filiament, thus regulating new filiament formation. [115] PtdIns(4,5)$P_2$ also binds to profilin ($K_D$ < 0.1μM) which inhibits actin polymerisation. [116] Thus PtdIns(4,5)$P_2$ may guide actin polymerisation by regulation of the gelsolin-actin and the profilin-actin complexes. It has also been suggested that there is a direct role for phosphoinositides in exocytosis from chromaffin cells. [117] It is the cytoskeletal element involved in exocytosis that is regulated by the phosphoinositides.

Preparations of α-actinin from striated muscle contain bound PtdIns(4,5)$P_2$. However, α-actinin from smooth and striated muscle bind exogenous PtdIns(4,5)$P_2$ with fairly high affinity. α-Actinin *in vitro* binds to F-actin and crosslinks actin filiaments, thereby increasing the viscosity of F-actin solutions. By adding PtdIns(4,5)$P_2$ to α-actinin/G-actin solutions from either muscle preparation increased the viscosity markedly. [118] The results indicate that PtdIns(4,5)$P_2$ is necessary for α-actinin to obtain maximum gelating activity.

Human brain contains an inositol lipid linked to glucosamine, mannose and ethanolamine. [119] PtdIns glycans serve to anchor proteins to the outer cell membrane, thus increasing lateral mobility. The ethanolamine residue forms an amide by linking to the terminal carboxyl group of the protein; there is a mannose-containing glycan and a monoacetylated glucosamine residue linked to the 6-position of *myo*-inositol *via* a glycosidic linkage. (For reviews see references [122,123]). In brain, cell surface glycoproteins, for example Thy-1, are anchored to the membrane *via* glycosyl PtdIns linkages, [124,125] and the expression of the glycoproteins may be regulated during development. Fouchier and coworkers [126] have demonstrated two types of PLC which are specific for glycosylated forms of PtdIns. The bulk of the hydrophobic *sn*-1,2 diacylglycerol moiety contains two long fatty acids linked to the *sn*-1 and *sn*-2 hydroxyl groups of glycerol. First, stearic acid, a $C_{18}$ saturated fatty acid linked to the *sn*-1 of glycerol. Second, arachidonic acid, a $C_{20}$ unsaturated fatty acid consisting of four *cis* double bonds linked to the *sn*-2 position of glycerol. (Figure 8). This is not the case in PtdIns anchors, where these fatty acid residues are replaced by myristate, octadecanol (as a 1-alkyl substituent), or docosanoate depending on the tissue source. The addition of nerve growth factor to PC12 phaeochromocytoma cells stimulated the production of [$^3$H]myristate-labelled species of diacylglycerol (but not for [$^3$H]arachidonic-diacylglycerol) and a *myo*-inositol phosphate glycan. [82] The latter may serve as an intracellular second messenger, which has been proposed for insulin action. [127]

## 2.6 Inositol Phosphate Receptors

Activation of PLC *via* G-proteins results in the formation of DAG and Ins(1,4,5)$P_3$ by the hydrolysis of PtdIns(4,5)$P_2$. Ins(1,4,5)$P_3$ elicits an increase in $Ca^{2+}$, released from non-mitochondrial stores. The release of calcium is specific for the D-isomer, is unaffected by known $Ca^{2+}$-channel blockers, requires the presence of $K^+$ and may occur even at low temperatures. (Ins(1,4,5)$P_3$ receptors are reviewed in [128-130]). The effects of Ins(1,4,5)$P_3$ on $Ca^{2+}$-release are mediated through intracellular receptors which were observed some time ago. The first demonstration of these sites was made in tissues such as liver and neutrophils. [131] The low levels of receptors present in these tissues precluded their direct characterisation. Higher levels of Ins(1,4,5)$P_3$ receptors were then found in the central nervous system (CNS) where they are present in high density in the Purkinje cells of the cerebellum. [132,133]

### 2.6.1 Purification and Characterisation of the Ins(1,4,5)$P_3$ Receptor

The Ins(1,4,5)$P_3$ receptor possesses intrinsic properties which reflect its biological function. For example, physiological levels of $Ca^{2+}$ inhibit the binding of Ins(1,4,5)$P_3$ to its receptor with an $IC_{50}$ of 300nM [134,135] which implies that the $Ca^{2+}$ released *via* the action of Ins(1,4,5)$P_3$ feeds back to inhibit further $Ca^{2+}$-release by Ins(1,4,5)$P_3$. Raising the intracellular pH from 7.8 to 8.5 causes a 3-fold increase in the binding of Ins(1,4,5)$P_3$ to its receptor.

The high concentration of the Ins(1,4,5)$P_3$ receptor in the endoplasmic reticulum (ER) of cerebellar Purkinje cells allowed Supattapone and coworkers to purify the receptor to homogeneity. [136] The potent inhibition of $Ca^{2+}$-release by heparin binding to the Ins(1,4,5)$P_3$ receptor [134,137-140] allowed the Ins(1,4,5)$P_3$ receptor to be purified on heparin-agarose affinity columns. A second purification step utilised concanavalin-A Sepharose chromatography, taking advantage of the glycosylation of the Ins(1,4,5)$P_3$ receptor. The combination of both steps allowed an overall 1000-fold purification of the receptor. The same purification was also achieved using an Ins(1,4,5)$P_3$ affinity column.

[141] When the protein was analysed by polyacrylamide gel electrophoresis (PAGE) it moved as a single 260kDa band. The native receptor is about 1000kDa which indicated a homotetramer [136] and has been confirmed by cross-linking studies. [142] Ins(1,4,5)$P_3$ induced $Ca^{2+}$-release may be positively cooperative, [143,144] indicating that the four subunits communicate with each other such that activation of one subunit increases the probability that another subunit will be activated. However, tritium-labelled Ins(1,4,5)$P_3$ binding to the purified receptor showed no evidence of cooperativity with a Hill coefficient of one. [136]

### 2.6.2 Regulation of Receptor Function and Calcium Release

Three different enzymes, cyclic AMP-dependent protein kinase (PKA), [145,146] protein kinase C [146] (PKC) and $Ca^{2+}$/calmodulin-dependent protein kinase (CAM-K-II) [146] may stoichiometrically phosphorylate a serine residue of the Ins(1,4,5)$P_3$ receptor, albeit at different sites of the polypeptide chain, (Figure 9). The peptide chain was phosphorylated by PKA at two serine residues, Ser-1589 and Ser-1755. [147] At low concentrations of

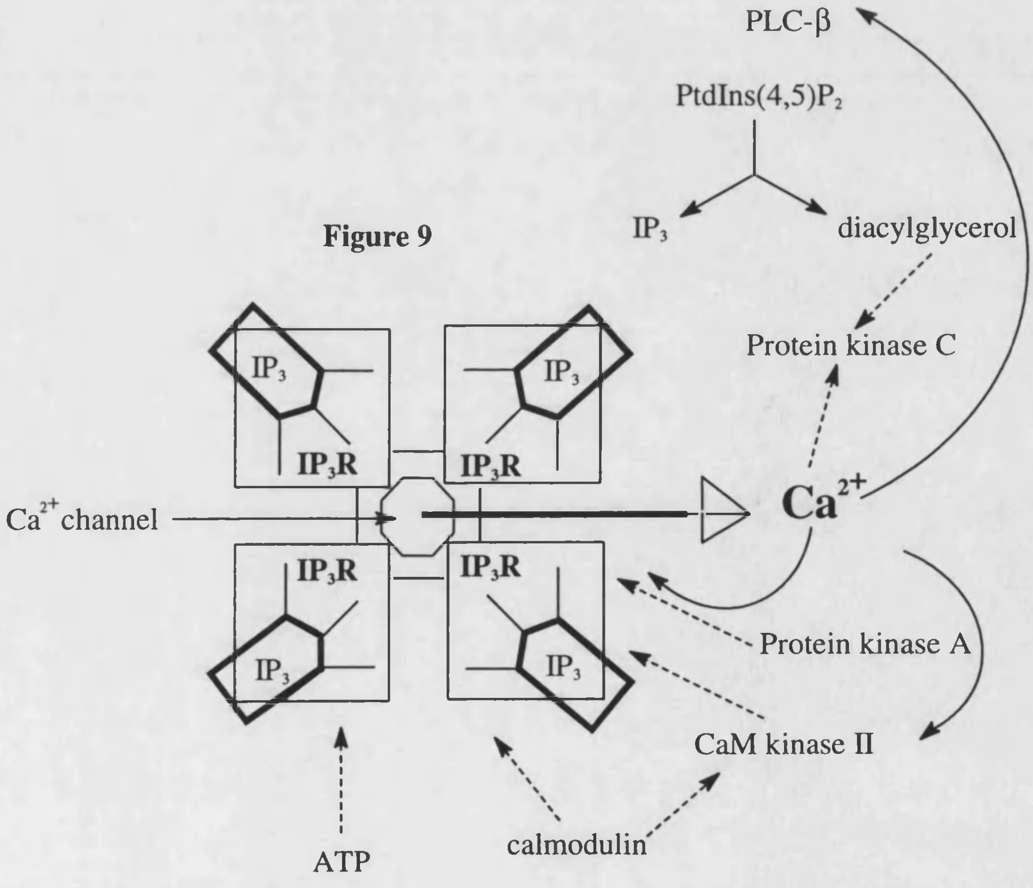



**Figure 9**

PKA, Ser-1755 was phosphorylated, but higher concentrations of this enzyme were required to phosphorylate the serine residue at position 1589. Following this work, Ferris and coworkers [148] also found that the Ins(1,4,5)$P_3$ receptor could autophosphorylate. Positive evidence has come from renaturation experiments in which autophosphorylation has been seen on nitrocellulose membranes following SDS-PAGE. [148] The Ins(1,4,5)$P_3$ receptor can also phosphorylate a synthetic peptide, indicating that the Ins(1,4,5)$P_3$ receptor has intrinsic protein kinase activity. This phosphorylation regulates the function of the Ins(1,4,5)$P_3$ receptor. Receptor phosphorylation by PKA in cerebellar cell membranes decreases the potency of Ins(1,4,5)$P_3$ to release $Ca^{2+}$. [145] Since the phosphorylation of the Ins(1,4,5)$P_3$ receptor by PKA stimulates the $Ca^{2+}$ pump, the endoplasmic reticulum $Ca^{2+}$ levels are increased so that the absolute amount of $Ca^{2+}$ released by Ins(1,4,5)$P_3$ is enhanced. [145] Cyclic AMP-dependent hormones increase Ins(1,4,5)$P_3$-induced $Ca^{2+}$-release in liver cells. [149] Phosphorylation of the Ins(1,4,5)$P_3$ receptor by CAM-K-II and PKC may provide some feedback regulation of the PtdIns cycle, following the production of DAG which stimulates the action of PKC, and release of $Ca^{2+}$ by Ins(1,4,5)$P_3$, which activates PKC and CAM-K-II. A cerebellar-specific form of CAM-K-II present in high concentrations in Purkinje cells [150-152] is found in a similar location to the Ins(1,4,5)$P_3$ receptor.

The Ins(1,4,5)$P_3$ binding protein also contains a calcium channel. Evidence for this has come from experiments using lipid vesicles containing the Ins(1,4,5)$P_3$ receptor in the reconstituted membrane. Experiments using lipid vesicles allowed $^{45}Ca^{2+}$-release by Ins(1,4,5)$P_3$ to be measured. [153] $Ca^{2+}$-release by Ins(1,4,5)$P_3$ reflects the affinity of Ins(1,4,5)$P_3$ at its binding sites. Heparin, an antagonist of Ins(1,4,5)$P_3$ binding and $Ca^{2+}$-release, demonstrated the Ins(1,4,5)$P_3$ receptor contained the Ins(1,4,5)$P_3$ recognition site and a $Ca^{2+}$-channel. [153]

ATP has been shown to regulate the Ins(1,4,5)$P_3$ receptor in an allosteric manner. [154] Between 1 and 10μM, ATP cooperatively enhances the ability of Ins(1,4,5)$P_3$ to release $Ca^{2+}$. This effect is biphasic, since increasing the concentration of ATP to physiological levels of 0.1-1.0mM diminishes the effect of Ins(1,4,5)$P_3$ on $Ca^{2+}$-release. At physiological concentrations of ATP (about 1mM), one would expect no obvious influence of this adenine nucleotide on the action of Ins(1,4,5)$P_3$. Once Ins(1,4,5)$P_3$ has released $Ca^{2+}$, the $Ca^{2+}$-dependent ATPase would be activated in order to replenish the $Ca^{2+}$ stores, which deplete ATP near the Ins(1,4,5)$P_3$ receptor. The ATP bound with low affinity will then dissociate and the release of $Ca^{2+}$ would be increased in a feed forward manner. When ATP concentrations are further depleted then the ATP bound with high affinity would dissociate and the release of $Ca^{2+}$ by Ins(1,4,5)$P_3$ would fall, possibly protecting the cell from harmful fluctuations of $Ca^{2+}$ levels. This model may also contribute to the spike-like changes in intracellular $Ca^{2+}$ concentrations associated with $Ca^{2+}$ oscillations [155-158] and help account for the marked cooperativity of Ins(1,4,5)$P_3$-induced $Ca^{2+}$-release. [159,160]

$Ca^{2+}$ release by Ins(1,4,5)$P_3$ in permeablised pancreatic acinar cells is a noncontinuous process, such that submaximal Ins(1,4,5)$P_3$ concentrations release submaximal amounts of Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores [161] even under conditions where the Ins(1,4,5)$P_3$ is not degraded. This activity may be described as "quantal" since subfractions of Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores are activated by Ins(1,4,5)$P_3$. The quantal release of $Ca^{2+}$ in purified reconstituted Ins(1,4,5)$P_3$ receptor indicate that the phenomenon is a property of the Ins(1,4,5)$P_3$ receptor. [162] $Ca^{2+}$ channels are not activated by submaximal Ins(1,4,5)$P_3$ concentrations in the reconstituted system. [162] The successive addition of Ins(1,4,5)$P_3$ provide an arithmetic increase in $Ca^{2+}$-release over a range of 10-200nM Ins(1,4,5)$P_3$. [162] $Ca^{2+}$ signalling is complex involving oscillations and waves, [156,158] so the sequential release properties of the Ins(1,4,5)$P_3$ receptor are critical for the regulation of $Ca^{2+}$ concentrations in all cells.

A recent study [163] has demonstrated a $Ca^{2+}$-induced degradation of the neuronal Ins(1,4,5)$P_3$ receptor which generated two fragments of 130 and 95kDa. The fragments retained immunoreactivity towards a C-terminal-specific antibody, so presumably they are derived from the part of the receptor where the $Ca^{2+}$-channel domain is located. The results may indicate that activation of the Ins(1,4,5)$P_3$ receptor, by causing an increase in intracellular $Ca^{2+}$ concentration may result in degradation of the N-terminal, Ins(1,4,5)$P_3$ binding receptor site. The Ins(1,4,5)$P_3$ receptor was shown to be an excellent substrate for a purified $Ca^{2+}$-activated enzyme from the calpain family which are nonlysosomal proteolytic enzymes, are active at physiological pH and require millimolar or micromolar calcium ions for activation. Calpains may be autolytically cleaved by $Ca^{2+}$ ions, thereby increasing the sensitivities of these enzymes so they become responsive to fluctuations of physiological $Ca^{2+}$ ion concentration. Thus, it is possible that the increase in $Ca^{2+}$ ion concentration could induce degradation of the Ins(1,4,5)$P_3$ $Ca^{2+}$-channel.

### 2.6.3 Cloned Ins(1,4,5)$P_3$ Receptors

The Ins(1,4,5)$P_3$ receptor was originally identified as a 400kDa cerebellar membrane glycoprotein designated $P_{400}$ localised to Purkinje cells [164] of normal mice, but was reduced in Purkinje cell-deficient mutants. $P_{400}$ is a glycoprotein with a molecular mass of 250kDa on SDS-PAGE and highly enriched in the cerebellum. The Ins(1,4,5)$P_3$ receptor gene was cloned and its amino acid sequence determined. [165] On the basis of its cDNA sequence, $P_{400}$ comprised 2749 amino acids with a molecular mass of 313kDa, a value greater than that obtained by SDS-PAGE. This difference in molecular mass may be due to either an aberrant electrophoretic migration of the protein or posttranslational proteolytic processing. Purified $P_{400}$ bound [$^3$H]Ins(1,4,5)$P_3$ in a saturable and specific manner analogous to that observed for the purified Ins(1,4,5)$P_3$ receptor. [166] Definitive proof that $P_{400}$ and the Ins(1,4,5)$P_3$ receptor are identical was obtained from experiments in which transfection of $P_{400}$ cDNA into NG108-15 cells resulted in the expression of Ins(1,4,5)$P_3$-binding sites. [165] The Ins(1,4,5)$P_3$ receptor is highly conserved among mammals, because the sequences in rodents and humans differ by less than 10% and only by 1% between rat and mouse.

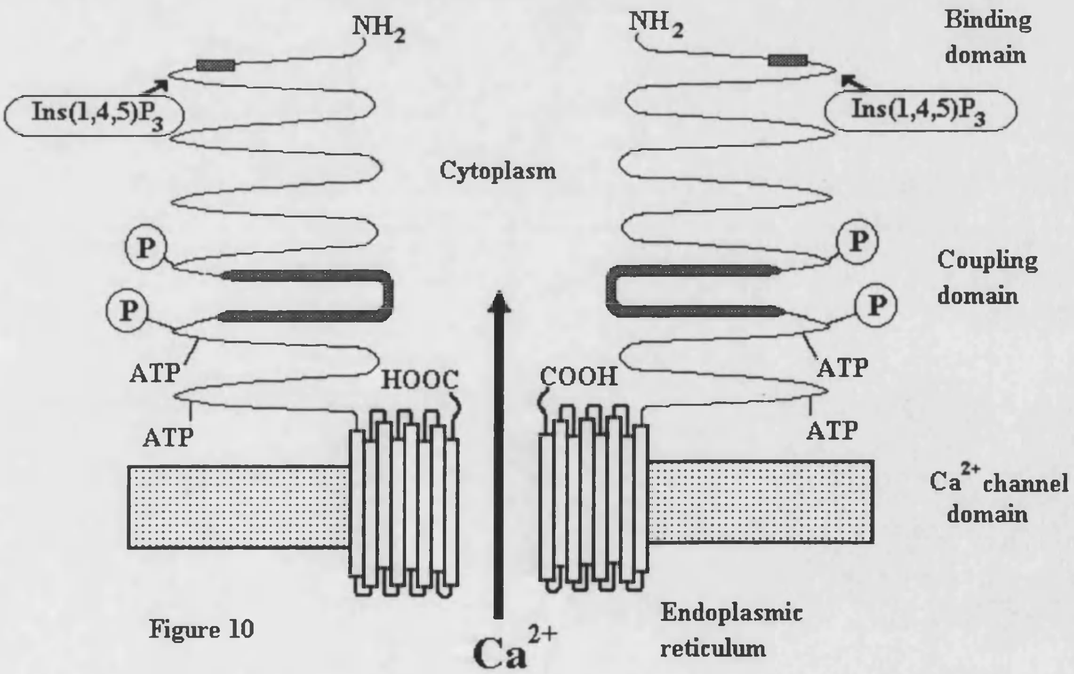



Figure 10

A model for the Ins(1,4,5)$P_3$ receptor is shown in Figure 10. A subunit consists of six or eight transmembrane domains. [165] In the eight subunit domain, the first four are flanked by net positive charges and the last four by net negative charges. The last four domains show closest homology with the ryanodine receptor, the $Ca^{2+}$-stimulated $Ca^{2+}$-release channel of muscle, so they are likely to comprise the $Ca^{2+}$-permeable pore.

Mutagenesis studies have shown that the Ins(1,4,5)$P_3$ binding site lies in the amino-terminal 400 amino acid residue since when this domain is deleted Ins(1,4,5)$P_3$ does not bind to the receptor. [165] Moreover, the receptor retaining only the N-terminal quarter of the molecule provides soluble peptides which are monomeric but retain the ability to bind. [165,166] The N-terminal peptide binds Ins(1,4,5)$P_3$ with substantially less affinity than does the full receptor protein, therefore the overall conformation of the intact protein may be important for physiological Ins(1,4,5)$P_3$ binding. If the Ins(1,4,5)$P_3$ binding site is present at the N-terminal end of the protein and the $Ca^{2+}$ channel is regarded as being at the extreme C-terminal, then Ins(1,4,5)$P_3$ binding must elicit a conformational change over a span of 1400 amino acids. Both sites for serine phosphorylation by PKA, [147] as well as the ATP binding sites [167] are located in the "coupling region" between domains for Ins(1,4,5)$P_3$ binding and the $Ca^{2+}$ channel, where these regulatory sites may affect the ability of Ins(1,4,5)$P_3$ to open the $Ca^{2+}$ channel.

Other receptors of the same family, derived from different genes have been identified. The complete sequences of three members of this family, designated type-1 [165] type-2 [168] and type-3 [169] have been reported as well as partial cDNA's for two other subtypes. [168,170] The original Ins(1,4,5)$P_3$ receptor is designated the type-1 receptor. The type-2 receptor has significant homology with type-1 receptor especially in Ins(1,4,5)$P_3$ binding and transmembrane domains. [168] The type-2 receptor has a significant higher affinity for Ins(1,4,5)$P_3$ than type-1 receptor. A specific tissue may also contain more than one different receptor-type, which raises the possibility that intracellular $Ca^{2+}$ signalling may involve multiple pathways having different regulatory properties on different receptor-type transduction pathways.

Receptor type-3 is 2670 amino acids in size and has 62% and 64% identity with type-1 and type-2 receptor respectively. The expression of recombinant rat type-3 receptor in COS-7 cells demonstrated its ability to bind Ins(1,4,5)$P_3$, Ins(1,3,4,5)$P_4$ and Ins(1,2,3,4,5,6)$P_6$. Other studies have shown the type-3 receptor to be wicely distributed in the endoplasmic reticulum. [169] The type-3 receptor is predominantly expressed in pancreatic islets over type-1 and type-2 indicating that type-3 may be responsible for initiating the effects of Ins(1,4,5)$P_3$ on insulin secretion. Finally, the type-3 receptor does not appear, at present, to be a substrate for the kinases that are responsible for phosphorylation of type-1 receptor.

A relative of the Ins(1,4,5)$P_3$ receptor is the ryanodine receptor. [165,171,172] Ryanodine is an alkaloid that binds to a protein responsible for CICR from the sarcoplasmic reticulum of striated muscle as a key component of excitation-contraction coupling. [173,174] Both Ins(1,4,5)$P_3$ and ryanodine receptors possess $Ca^{2+}$ channels to promote $Ca^{2+}$-release. Both types of receptor are large homotetrameric proteins and the tetramer of the receptor binds three to four molecules of Ins(1,4,5)$P_3$. [159] ATP induces $Ca^{2+}$-release in the ryanodine receptor, but in the Ins(1,4,5)$P_3$ receptor, ATP enhances the stimulation of $Ca^{2+}$-release by Ins(1,4,5)$P_3$. Ryanodine receptors are concentrated in skeletal and cardiac muscle, whereas Ins(1,4,5)$P_3$ receptors are at their highest levels in smooth muscle and brain.

## 2.7 The Role of $Ca^{2+}$ in the Cell

When life started, the oceans were more alkaline than at present, with micromolar concentrations of $Ca^{2+}$. [175] A phosphate-based metabolism would have been difficult if $Ca^{2+}$ concentrations were higher. In eukaryotes, the $Ca^{2+}$ concentration is several orders of magnitude higher in the extracellular space than in the cytoplasm. This situation of $Ca^{2+}$ concentration arose through evolution, in order to protect phosphate-based metabolism in cells, because high concentrations of calcium would cause calcium phosphate solubility products to be exceeded. During evolution has seen how concentration gradients of calcium have been exploited to provide a rapid intracellular signalling mechanism.

Ringer, in 1883 [176] was the first to publish an effect of calcium on cell function. He observed that cardiac muscle could not continue to beat *in vitro* without a minimum concentration of extracellular calcium. Much later other groups [177,178] discovered new roles for $Ca^{2+}$ in a variety of cell types. The signalling function of this cation is brought about by the interaction of specific $Ca^{2+}$-binding proteins with low calcium concentrations, resulting in phosphorylation, or enzyme activation, depending on the target for $Ca^{2+}$-binding protein. [179,180] Other aspects of $Ca^{2+}$ in cell function include fertilization of oocytes, control of cell growth, beating of the heart, transmission of nerve impulses, contraction of skeletal and vascular muscle, the clotting of blood, and the secretion of hormones and digestive enzymes.

### 2.7.1 Regulation of Intracellular $Ca^{2+}$ An Overview

Cells maintain a basal cytosolic free $Ca^{2+}$ concentration $[Ca^{2+}]_i$ in the order of 50-200nM, whereas the extracellular concentration of $Ca^{2+}$ is normally in the millimolar range. This occurs by active extrusion of $Ca^{2+}$ across the plasma membrane and sequestration of $Ca^{2+}$ within the cell by pumping $Ca^{2+}$ into intracellular compartments or by binding of $Ca^{2+}$ to structures such as cytosolic proteins. There are two mechanisms for removing $Ca^{2+}$ from the cell, both of them involve the plasma membrane. First, ATP fuels a $Ca^{2+}$-ATPase and pumps $Ca^{2+}$ out of the cell. [181,184] Second, at the expense of a $Na^+$ ion gradient, intracellular $Ca^{2+}$ is removed by the cell in a $Na^+/Ca^{2+}$ exchanger. [185] These mechanisms offset the continued passive influx of $Ca^{2+}$ into the cytoplasm from the extracellular space. The leakage of $Ca^{2+}$ occurs through specialised channels, present in the plasma membrane. Also present are intracellular compartments which sequester $Ca^{2+}$ *via* $Ca^{2+}$-pumping mechanisms. These compartments include the endoplasmic reticulum and mitochondria. [186] Specialised $Ca^{2+}$-sequestering organelles exist which are cell specific; these include the sarcoplasmic reticulum of muscle fibres [187] and the dense tubular system of platelets. A $Ca^{2+}$ channel is located in the membranes of the intracellular organelles which in some instances may be controlled by certain small molecules such as Ins(1,4,5)$P_3$, releasing $Ca^{2+}$ into the cytoplasm.

### 2.7.2 $Ca^{2+}$ Regulation *via* Ins(1,4,5)$P_3$

The mobilisation of $Ca^{2+}$ ions via the phosphoinositide signalling pathway involves two phases. First, $Ca^{2+}$-release from an intracellular store and second, a longer phase of extracellular $Ca^{2+}$ entry. [188,189] In the absence of extracellular $Ca^{2+}$, agonist stimulation *via* the phosphoinositide signalling pathway results in a transient increase in $[Ca^{2+}]_i$, originating from the release of a finite intracellular $Ca^{2+}$ pool. When physiological (mM) extracellular $Ca^{2+}$ is present, the elevation in $[Ca^{2+}]_i$ is sustained due to the additional component of extracellular $Ca^{2+}$ influx. Extracellular $Ca^{2+}$ influx is also the source for refilling of the intracellular $Ca^{2+}$ pool upon termination of receptor activation.

### 2.7.3 $Ca^{2+}$-Release

The early studies of $Ca^{2+}$ release by Ins(1,4,5)$P_3$ identified the $Ca^{2+}$-release pool as the endoplasmic reticulum (ER) because $Ca^{2+}$-release occurred in the presence of toxins which inactivate the mitochondrial. Subsequently, the $Ca^{2+}$-release pool was thought to reside in a highly specialised component of the ER, or a unique $Ca^{2+}$-releasing organelle termed the "calciosome". [190] The evidence for supporting either the ER or the calciosome or both comes from two experimental approaches: first, analysis of the functional organisation of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool: second, the identification of its location within the cell and its relation to other cellular compartments.

Only a fraction of the total $Ca^{2+}$ is released by Ins(1,4,5)$P_3$ in permeabilised cells. This may indicate segragation of some of the Ins(1,4,5)$P_3$-regulated $Ca^{2+}$-release sites from the entirety of the $Ca^{2+}$ uptake and storage capacity available within the cell. It may be possible that some of the nonmitochondrial Ins(1,4,5)$P_3$-insensitive $Ca^{2+}$ pools (for example CICR) are also involved in $Ca^{2+}$ signalling. This segregation may in part be an artifact of the permeabilisation process. [191-193] When cells are permeabilised the apparent communication mechanism between Ins(1,4,5)$P_3$-sensitive and Ins(1,4,5)$P_3$-insensitive $Ca^{2+}$ pools is disrupted. GTP can cause a link between the Ins(1,4,5)$P_3$-sensitive and insensitive $Ca^{2+}$ pools in several permeabilised systems. A segregation of $Ca^{2+}$-release and uptake sites has also been observed in rat parotid cells under circumstances [194] where $Ca^{2+}$-release was induced by the 5-phosphatase resistant analogue of Ins(1,4,5)$P_3$, *myo*-inositol-1,4,5-trisphosphorothioate, Ins(1,4,5)$PS_3$ was rapidly resequested into the Ins(1,4,5)$P_3$-sensitive pool. The compartment involved in $Ca^{2+}$-release was structurally different from the compartment within the pool for the reuptake of $Ca^{2+}$. In the presence of Ins(1,4,5)$P_3$ there was no substantial reuptake into the Ins(1,4,5)$P_3$-sensitive pool. Thus, it may be concluded that a kinetic delay in translocation of $Ca^{2+}$ from uptake to release sites was caused or exaggerated by cell permeabilisation. This may result from distruction of the translocation of $Ca^{2+}$ between the uptake and release sites. It should be noted that GTP has no effect on the kinetics of translocation of $Ca^{2+}$ between the uptake and release sites in the parotid cells, [194] indicating that different mechanisms are involved in this system. These two lines of experimental evidence suggest there is at least some separation of Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$-release and uptake sites. This separation is reminiscent of that attributed to skeletal muscle sarcoplasmic reticulum (SR) [195] and, based on the observed effects of GTP in various permeabilised cells, it is likely to be a general phenomenon in nonmuscle cells as well.

### 2.7.4 Capacitive $Ca^{2+}$ Entry

Ins(1,4,5)$P_3$ binds to its receptor to release $Ca^{2+}$, followed by a subsequent entry of $Ca^{2+}$ from the extracellular space. However, Ins(1,4,5)$P_3$ may not directly activate the entry of $Ca^{2+}$ since application of Ins(1,4,5)$P_3$ to plasma membranes has no effect on $Ca^{2+}$ permeability. [196-198] Thus, it appears that $Ca^{2+}$ entry is activated by the emptying of the Ins(1,4,5)$P_3$-sensitive pool. [199] When phosphoinositide turnover takes place, the $Ca^{2+}$ pool is emptied and the pathway for $Ca^{2+}$ entry from the extracellular space is open. This mechanism has been termed "capacitative $Ca^{2+}$ entry" [199] and is supported by several pieces of experimental evidence.

### 2.7.5 $Ca^{2+}$ as a Signal for $Ca^{2+}$ Entry

It has been demonstrated that that $Ca^{2+}$ flows directly into the pool during refilling using the $Ca^{2+}$ indicator fura-2. This has been used to monitor changes in cytosolic $Ca^{2+}$ in acinar and parotid cell types. [200-202] A piece of evidence to support the capacitative model was shown in parotid cells, which were stimulated with the muscarinic agonist methacholine, in a $Ca^{2+}$-free medium, followed by treatment with the receptor antagonist atropine and extracellular $Ca^{2+}$ to produce an elevation of $[Ca^{2+}]_i$. The elevation of $[Ca^{2+}]_i$ produced under these conditions was larger than that produced by the addition of $Ca^{2+}$ to unstimulated cells in a $Ca^{2+}$-free medium. The elevation of $[Ca^{2+}]_i$ on the addition of $Ca^{2+}$ was seen 20min after the addition of atropine, although it is not seen if the intracellular stores were first allowed to refill. It was also discovered that thapsigagin [203] (the nonphorbol ester tumour promotor) releases intracellular $Ca^{2+}$ by a mechanism which eliminates the need for phosphoinositide turnover. [202] Thapsigargin is a toxin which depletes intracellular $Ca^{2+}$ stores by a specific action on the $Ca^{2+}$-ATPase on intracellular membranes; it does not, however, inhibit active $Ca^{2+}$ transport by the plasma membrane of cells. [203] In the presence of extracellular $Ca^{2+}$ this agent induces a sustained increase in $[Ca^{2+}]_i$, which in turn depends on extracellular $Ca^{2+}$; in other words, it results from $Ca^{2+}$ entry. However, when the muscarinic agonist methacholine, is added to cells during $Ca^{2+}$ entry induced by thapsigargin, only a small transient increase in $[Ca^{2+}]_i$ was achieved, which then returned to the level produced by thapsigargin alone. This indicated that the $Ca^{2+}$ entry induced by the toxin is not additive with that induced by the agonist. Methacholine and thapsigargin thus appear to activate the same mechanism for $Ca^{2+}$ entry.

Another study [204] has examined divalent cation entry into single human umbilical vein endothelial cells. Earlier it was demonstrated that histamine and thrombin stimulate $Ca^{2+}$ entry into these cells. [205,206] In such studies, manganese ions ($Mn^{2+}$) were used which bind to fura-2 with high affinity and quench its fluorescence, so that the rate of quench of fluorescence of intracellular fura-2 when $Mn^{2+}$ is present extracellularly is taken as a measure of surface membrane divalent cation permeability. In many cell types $Mn^{2+}$ enters through the same channels as $Ca^{2+}$. [207] When $Mn^{2+}$ is added during histamine receptor stimulation, $Mn^{2+}$ entry coincides with intracellular $Ca^{2+}$-release. When histamine stimulation occurs to deplete intracellular stores and $Mn^{2+}$ is added, then $Mn^{2+}$ entry also occurs. However, if external $Ca^{2+}$ is added (allowing the stores to refill) before the addition of $Mn^{2+}$ then there is no $Mn^{2+}$ entry, indicating that $Mn^{2+}$ only enters the cell if the $Ca^{2+}$ stores are depleted. It was found that the rate of $Mn^{2+}$ entry varies inversely with the $Ca^{2+}$ content of the intracellular stores. These results do not reveal a mechanism for this phenomenon. The emptying of the intracellular stores may influence the permeability of the plasma membrane at some distance in which case it may be considered that this communication could be accomplished through some kind of intracellular signalling.

### 2.7.6 Metabolites of Cytochrome P-450

There is recent evidence indicating that the binding of Ins(1,4,5)$P_3$ followed by $Ca^{2+}$ release is not a sufficient stimulus for initiating $Ca^{2+}$ influx. One such finding suggests that a product of the oxidative cytochrome P-450 enzyme system somehow produces a messenger for $Ca^{2+}$ entry. [208,209] Several compounds that inhibited cytochrome P-450 were also found to inhibit $Ca^{2+}$ entry and therefore provided a correlation between the two inhibitory activities appears to exist. [210-212] However, these compounds also inhibit $Ca^{2+}$-activated $K^+$ channels. [213] $Ca^{2+}$ entry and $Ca^{2+}$-activated $K^+$ channels are regulated by different mechanisms, therefore the compounds may block the $Ca^{2+}$ entry channels themselves, rather than the formation of the unknown messenger which activates the channel.

### 2.7.7 The Possible Role of $Ins(1,3,4,5)P_4$ and $Ins(1,3,4,6)P_4$ in $Ca^{2+}$ Influx

Two *myo*-inositol tetrakisphosphates, namely $Ins(1,3,4,5)P_4$ and $Ins(1,3,4,6)P_4$ have longer half-lives in cells than $Ins(1,4,5)P_3$ and may have a direct role in $Ca^{2+}$ regulation because both compounds are capable of $Ca^{2+}$ release from the $Ins(1,4,5)P_3$ sensitive stores. [214-218] The efficacy of both compounds however, is much lower than that of $Ins(1,4,5)P_3$, which may indicate that the major role for these two compounds is not as a mobiliser of $Ca^{2+}$ ions. However, there is evidence that $Ins(1,3,4,5)P_4$ may somehow act in concert with $Ins(1,4,5)P_3$. [219,220] This hypothesis is the subject of intense discussion. One piece of evidence has refuted it, [221] but the converse suggestion has been made that in cells which overexpress the 3-kinase enzyme, the accumulation of $Ins(1,3,4,5)P_4$ may be a negative regulator of the $Ca^{2+}$-phosphoinositide signal transduction pathway. [222]

Several pieces of experimental evidence have contributed towards explaining the confusion. First, in *Xenopus* oocytes, there appears to be a marked degree of synergism between $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ in controlling $Ca^{2+}$ entry. [223] Second, several reports have indicated that emptying the $Ins(1,4,5)P_3$-sensitive $Ca^{2+}$-stores is not a sufficient stimulus for initiating $Ca^{2+}$ influx. [224-226] Third, in the absence of $Ins(1,4,5)P_3$ it has been demonstrated that $Ins(1,3,4,5)P_4$ alone can activate a low conductance (2.5 picosiemens) divalent cation channel in inside-out patches prepared from endothelial cell plasma membranes.

It has also been demonstrated that high concentrations of $Ins(1,4,5)P_3$ (>100µM) and the non-phosphorylatable $Ins(2,4,5)P_3$, caused mobilisation and influx of $Ca^{2+}$ ions in the absence of $Ins(1,3,4,5)P_4$. [221] A report demonstrated a $Ca^{2+}$-induced $Cl^-$ current [224] whose regulation mirrored the behaviour described previously. [221] Another group [225] has shown that $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ can independently activate $Ca^{2+}$-release from crude microsomal preparations of lymphoid cell lines, indicating the presence of sub-populations of $Ins(1,4,5)P_3$- and $Ins(1,3,4,5)P_4$-sensitive vesicles. $Ca^{2+}$ was released from enriched plasma membranes from U937 cells by low concentrations of $Ins(1,3,4,5)P_4$, but only by much higher concentrations of $Ins(1,4,5)P_3$. This effect of $Ins(1,3,4,5)P_4$ was lost in the presence of detergents which was interpreted as showing that $Ins(1,3,4,5)P_4$ induced a $Ca^{2+}$ influx across the plasma membrane in the absence of $Ins(1,4,5)P_3$, perhaps by a specific $Ins(1,3,4,5)P_4$-activated ion channel. This work has been reviewed in two references. [227,228]

### 2.7.8 Calcium Influx Factor (CIF)

The concentration of $Ca^{2+}$ outside the cell is some 10,000 times higher than inside the cell. When Ins(1,4,5)$P_3$ binds to its receptor $Ca^{2+}$ is released from intracellular stores, however, what is not known is how the empty $Ca^{2+}$ stores signal to the plasma membrane allowing the intracellular stores to be refilled.

Two pieces of evidence, the first by Randriamampita and Tsien [229] indicated that a novel molecule gated this entry mechanism, at least in certain cell types. This group partially characterised an anionic compound with a molecular mass less than 500, which had hydroxyl, and/or amino groups, on adjacent carbons, together with at least one phosphate group. This molecule has been named calcium influx factor (CIF). Calcium influx factor is produced (in an unknown manner) by cells whose $Ca^{2+}$ stores have been depleted. It appears that CIF moves from organelles to cytoplasm and across the cell membrane into the supernatant where it may stimulate adjacent cells. A group of cross-desensitisation experiments has demonstrated that CIF is not a known second messenger.

A second piece of evidence by Parekh, Terlau and Stühmer [230] independently supports the main aspects of CIF. They used *Xenopus* oocytes which express exogenous serotonin receptors (5-$HT_{1C}$) and it was demonstrated that an $I_{CRAC}$-like current is enhanced by okadaic acid. Okadaic acid is a phosphatase inhibitor: thus the removal of a covalently bound phosphate by an okadaic acid-sensitive phosphatase may inactivate the messenger or protein that that has become phosphorylated as a result of the action of the messenger. This result agrees with the previous finding of Randriamampita and Tsien, [229] in so much as the CIF contains at least one phosphate group and the activity of this messenger is lost when the phosphate group is removed.

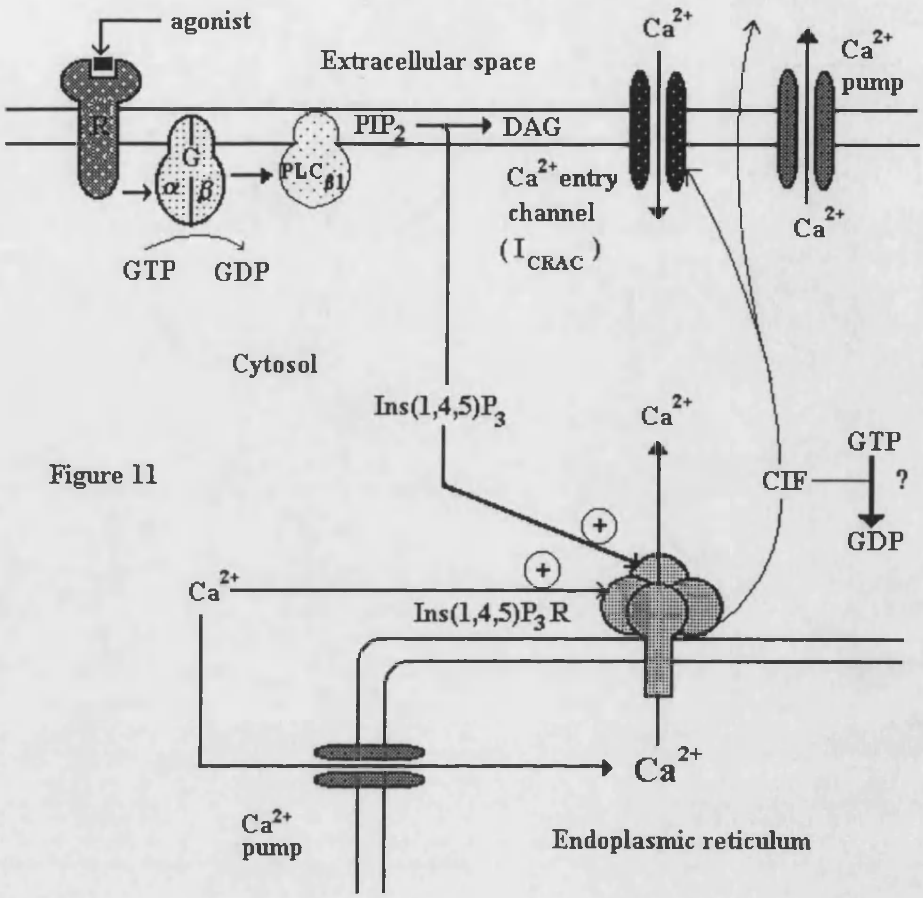



Figure 11

CIF is released from an intracellular organelle when the stores of $Ca^{2+}$ are depleted. The CIF opens influx pathways, presumably ion channels that allow $Ca^{2+}$ back into the cytoplasm where $Ca^{2+}$-ATPase transporters pump it back into the cell's stores, (Figure 11). The cell also uses other proteins to refill $Ca^{2+}$ stores such as voltage-activated $Ca^{2+}$-channels and exchangers. In the last few years the ion channel responsible for a certain current named the calcium release-activated current $I_{CRAC}$ [231,232] has been identified. The current has a very low single-channel conductance (about 20 femtosiemens), has a high selectivity for $Ca^{2+}$ ions over other divalent cations, and little or no voltage dependence for channel opening. The equilibrum potential for $Ca^{2+}$ is +150 millivolts, so the more hyperpolarised the cell the greater the influx of $Ca^{2+}$ through the $Ca^{2+}$ channel.

Two papers which followed on from the discovery of CIF, presented evidence [231,232] for at least one GTP-dependent step for $Ca^{2+}$ influx. Two GTP analogues, GTPβS and GTPγ S both inhibit $Ca^{2+}$ influx, [233,234] and this process somehow involves a GTP-binding protein and the hydrolysis of GTP rather than simple binding of GTP, which occurs with heterotrimeric G-proteins, and small G-proteins, such as *ras*. It is known that the small protein *rab* requires the hydrolysis of GTP: [233] so a protein such as this may be the signal suggested by the work of Parekh and coworkers. [230] However, if this messenger is a diffusable signal then its release or its action at the plasma membrane could involve the hydrolysis of GTP.

### 2.7.9 Tyrosine Kinases and Calcium Influx

Several reports have suggested a link between phosphorylation of tyrosine residues and $Ca^{2+}$ influx. [235-237] Tyrosine phosphorylation of platelet proteins occurred on stimulation with thrombin and thapsigargin and this appeared to result from depletion of intracellular $Ca^{2+}$ stores, rather than a rise in cytoplasmic $Ca^{2+}$. [235] $Ca^{2+}$ influx was inhibited in platelets using two different inhibitors of tyrosine kinases. [236] Again in fibroblasts, three different tyrosine kinase inhibitors blocked tyrosine phosphorylation and $Ca^{2+}$ influx to the PLC-linked hormone bradykinin and also blocked $Ca^{2+}$ influx due to the $Ca^{2+}$-ATPase inhibitor, thapsigargin. Future work will hopefully clarify the position concerning tyrosine phosphorylation and $Ca^{2+}$ influx.

## 2.8 Calcium Oscillations and Waves

Oscillations of $[Ca^{2+}]_i$ have been observed in many cell types in response to agonists. The nature of the oscillations varies among different cell types and for a given type of cell, the shapes of $Ca^{2+}$ transients may depend on which agonist induces the oscillations. [238,239]

There are two types of $Ca^{2+}$ oscillations: first, a sinusoidal fluctuation in $[Ca^{2+}]_i$ that occurs on top of a sustained elevation in $[Ca^{2+}]_i$. It was observed that this type of oscillation occurs following muscarinic receptor stimulation. These oscillations occur at low agonist concentrations and an increase of agonist causes an increase in amplitude of oscillation with little change in frequency. These oscillations usually diminish in magnitude with time suggesting a lack of feed forward mechanism to sustain them. The second type of oscillation is characterised by discrete spikes in $[Ca^{2+}]_i$. [239,240] The spikes occur with a variety of PLC-linked agonists. The $[Ca^{2+}]_i$ in the intervening periods between the $Ca^{2+}$ transients remain at or close to basal level. Spiking occurs at low agonist concentration, however the effect of the agonist is to increase the frequency of oscillation, not the amplitude. These oscillations may be sustained for long periods, indicating a feed forward mechanism that serves to regenerate each spike. Above a certain threshold of stimulation only a single spike occurs, which may not be followed by a sustained nonoscillating elevation of $[Ca^{2+}]_i$.

The mechanisms of $[Ca^{2+}]_i$ oscillation in cells is still speculative. Oscillations in the production of Ins(1,4,5)$P_3$ may produce the periodic release of $Ca^{2+}$. The periodic formation of Ins(1,4,5)$P_3$ may occur based on a negative feedback loop operating through protein kinase C [241] which reversibly phosphorylates a G-protein and inhibits the transduction of receptor binding to PLC activation. An alternative may be the reciprocal coupling between $Ca^{2+}$ and Ins(1,4,5)$P_3$ [242] resulting in spiking. In this model the production of Ins(1,4,5)$P_3$ leads to a rapid increase of $Ca^{2+}$ that positively feeds back to increase PLC activity and provide the cell with a burst of Ins(1,4,5)$P_3$. The $[Ca^{2+}]_i$ is lowered by reuptake of the $Ca^{2+}$ into the Ins(1,4,5)$P_3$-sensitive store which removes this positive feedback on PLC, thus Ins(1,4,5)$P_3$ levels fall.

Other mechanisms have been proposed in order to explain $Ca^{2+}$ oscillations in photoreceptors [243] and *Xenopus* oocytes. [244] Another model [238] necessitates the existence of a $Ca^{2+}$-releasable $Ca^{2+}$ pool. $Ca^{2+}$ released from an Ins(1,4,5)$P_3$-sensitive pool would be taken into a second $Ca^{2+}$ pool which is Ins(1,4,5)$P_3$ insensitive when $Ca^{2+}$ reaches a threshold level, a release channel opens and $Ca^{2+}$ is released back into the cytosol. In the presence of Ins(1,4,5)$P_3$ the process continues cyclically as long as the $Ca^{2+}$ concentration in the second pool manages to reach the threshold required for release.

**2.9 The Metabolism of Inositol Phosphates**

More than twenty of the possible sixty three *myo*-inositol phosphate isomers have been identified in tissues. Now that it has been established that Ins(1,4,5)$P_3$ acts as a second messenger in the mobilisation of $Ca^{2+}$, the routes of enzymatic metabolism and the regulation of the products together with the specific kinases and phosphatases are of considerable importance. Therefore, as well as the synthesis of Ins(1,4,5)$P_3$ in the cell, its degradation is also important to provide homeostasis within the cell. Ins(1,4,5)$P_3$ may be deactivated in one of two ways, (Figure 12). First, a 5-phosphatase removes the 5-phosphate group from Ins(1,4,5)$P_3$ to give *myo*-inositol 1,4-bisphosphate, Ins(1,4)$P_2$ which is inactive in stimulating $Ca^{2+}$-release. It has been demonstrated that Ins(1,4)$P_2$ is an allosteric activator of 6-phosphofructo-1-kinase [245] and has been reported to activate DNA polymerase-$\alpha$. [246] Ins(1,4,5)$P_3$ can also be phosphorylated by a 3-kinase to give

$Ins(1,3,4,5)P_4$, and both products can be regarded as "off" signals. The $Ins(1,4)P_2$ resulting from 5-phosphatase action on $Ins(1,4,5)P_3$ is further dephosphorylated to *myo*-inositol 4-phosphate, Ins(4)P *via* the action of a $Mg^{2+}$-dependent *myo*-inositol polyphosphate 1-phosphatase. [247-249] This enzyme has been purified from brain cytosol, has a molecular mass of 40-44,000Da and is inhibited by $Ca^{2+}$ and uncompetitively by $Li^+$. There is also some evidence that less than 5% of $Ins(1,4)P_2$ undergoes dephosphorylation to *myo*-inositol 1-phosphate, Ins(1)P, which suggests that a 4-phosphatase can slowly hydrolyse $Ins(1,4)P_2$. The activity of this enzyme is only observed in homogenates with very high concentrations (100-700μM) of $Ins(1,4)P_2$ even in the presence of $Li^+$. [250,251]

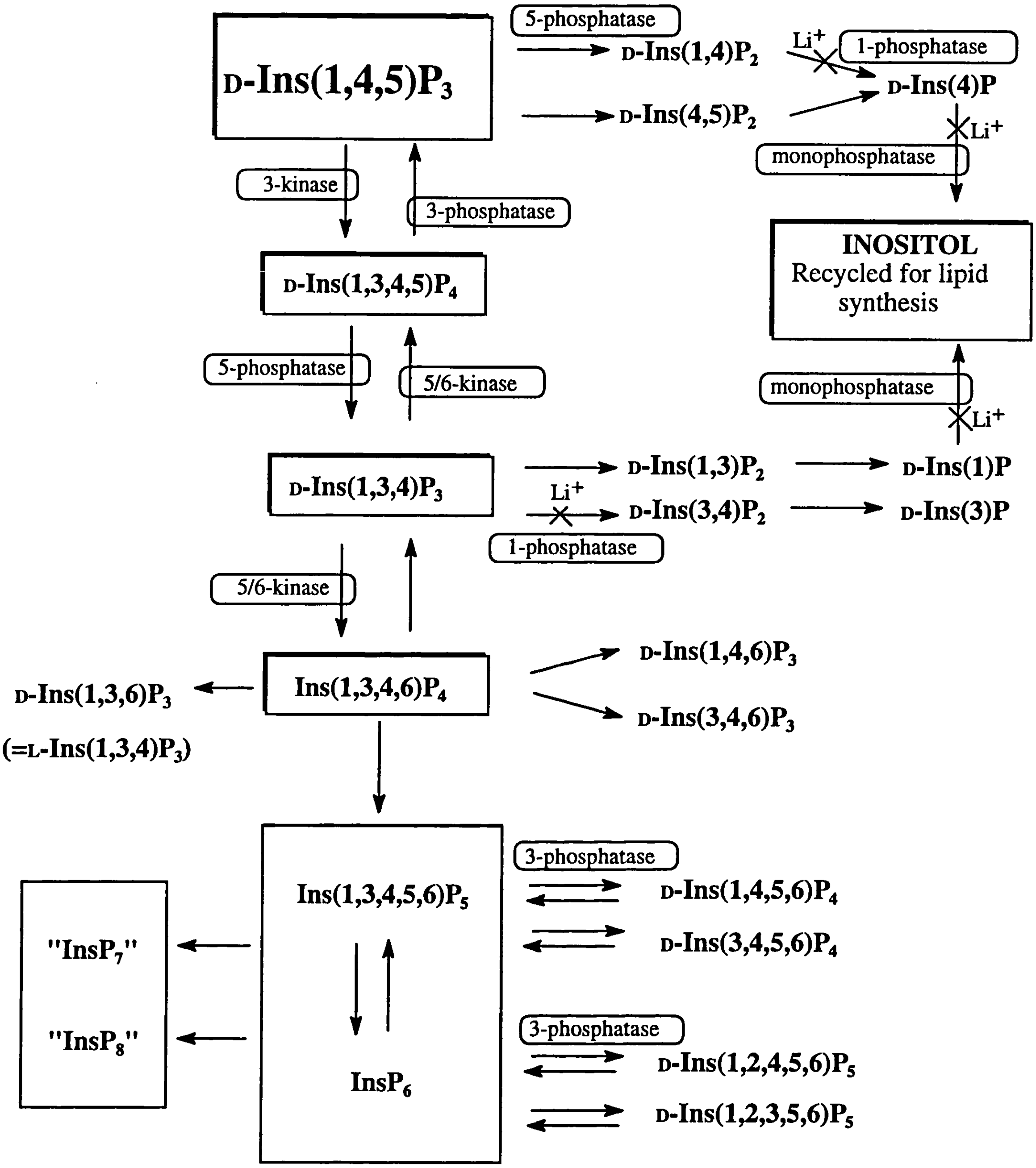



**Figure 12**

The major route of Ins(1,3,4,5)$P_4$ metabolism is the dephosphorylation by the 5-phosphatase to provide the cell with Ins(1,3,4)$P_3$. However, a $Li^+$ insensitive 3-phosphatase is present in the brain cytosol, which results in the reformation of Ins(1,4,5)$P_3$. [252,253] Ins(1,3,4)$P_3$ is further metabolised by *myo*-inositol polyphosphate 1-phosphatase [247-249] to produce Ins(3,4)$P_2$. Ins(1,3,4)$P_3$ may be dephosphorylated by the

$Li^+$ insensitive 4-phosphatase to give Ins(1,3)$P_2$. These two compounds, Ins(3,4)$P_2$ and Ins(1,3)$P_2$ are then further dephosphorylated by the 4- and 3-phosphatases respectively to give Ins(3)P and Ins(1)P. The enzyme, *myo*-inositol monophosphatase has similar affinities for the dephosphorylation of Ins(1)P and Ins(4)P and can dephosphorylate at the 3- and 5-positions for monophosphates. The enzyme has been purified to homogeneity. [254,255] The protein is a dimer, is inhibited by $Li^+$ in an uncompetitive manner, ($K_i$ <1nM) and the cDNA indicated it has 277 amino acids and is unrelated to any other known protein. [256]

### 2.9.1 Higher Inositol Phosphates

A interesting aspect of the metabolism of *myo*-inositol phosphates is the 3-kinase pathway which phosphorylates Ins(1,4,5)$P_3$ to give Ins(1,3,4,5)$P_4$. The majority of the Ins(1,3,4,5)$P_4$ is dephosphorylated to Ins(1,3,4)$P_3$ which may be then further phosphorylated by a 6-kinase to provide Ins(1,3,4,6)$P_4$. [257] The 6-kinase enzyme is widely distributed and has a $K_m$ of 29μM for Ins(1,3,4)$P_3$. The activity is unaffected by $Ca^{2+}$ or by $Li^+$ [258] but is inhibited by 10μM Ins(1,3,4,5)$P_4$. [259] The symmetrical tetrakisphosphate, Ins(1,3,4,6)$P_4$ is metabolised slowly in comparison to Ins(1,4,5)$P_3$, arguing against a role in $Ca^{2+}$-release although it can mobilise $Ca^{2+}$ in *Xenopus* oocytes [217] and neuroblastoma cells. [216] It may be possible that Ins(1,3,4,6)$P_4$ is the major precursor to the higher *myo*-inositol phosphates Ins(1,3,4,5,6)$P_5$ and phytic acid, Ins(1,2,3,4,5,6)$P_6$. [260] It appears that the phosphorylation of Ins(1,3,4,6)$P_4$ is rate-limiting and proceeds at maximum rates even when the PLC-linked receptors are not stimulated and thus are not regulated by PLC-linked agonists.

Another *myo*-inositol phosphate, Ins(3,4,5,6)$P_4$ is derived from Ins(1,3,4,5,6)$P_5$ and its formation is dramatically increased by PLC-linked agonists. [260] Agents such as thapsigargin and phorbol myristate acetate that by-pass PLC activation do not mimic agonist activation by increasing levels of Ins(3,4,5,6)$P_4$. Thus the reaction is regulated by a mechanism other than a Protein Kinase C/$Ca^{2+}$-signalling pathway. The levels of Ins(1,3,4,5,6)$P_5$ are not reduced by agonists and are high in most cells, indicating that Ins(3,4,5,6)$P_4$ may provide some function which as yet is unknown.

Within the last few years further discoveries in the *myo*-inositol polyphosphate field have provided *myo*-inositol polyphosphates more polar than Ins(1,2,3,4,5,6)$P_6$. [261-263] These compounds are pyrophosphorylated derivatives of Ins(1,3,4,5,6)$P_5$ and phytic acid and are present in AR4-2J pancreatoma cells [262] and in the slime mould *Dictyostelium.* [263] These pyrophosphate compounds are synthesised in an ATP-dependent manner from the respective *myo*-inositol polyphosphates. Enzymes are also present to dephosphorylate them back to the precursors and the standard free energy of hydrolysis of the pyrophosphate bond is in the region of 6.5–6.6kcal $mol^{-1}$, which is a similar value to the hydrolysis of pyrophosphate bonds of ATP. The function of these compounds is yet to be established. There is no evidence at present to show that these pyrophosphorylated derivatives are under the control of hormones or neurotransmitters.

### 2.9.2 Functions of Ins(1,3,4,5,6)$P_5$ and Ins(1,2,3,4,5,6)$P_6$

The two *myo*-inositol polyphosphates Ins(1,3,4,5,6)$P_5$ and Ins(1,2,3,4,5,6)$P_6$ are found in most cells in a micromolar to millimolar concentration range. Phytic acid can act as an enzyme inhibitor [264] and an antioxidant. [265] Phytic acid can increase the oxygen affinity for haemoglobin in a similar fashion to 2,3-diphosphoglycerate. [266,267] Ins(1,3,4,5,6)$P_5$ and Ins(1,2,3,4,5,6)$P_6$ but no other *myo*-inositol phosphates can lower blood pressure and decrease the heart rate in rat. [268,269] Ins(1,2,3,4,5,6)$P_6$ has also been reported to elicit $Ca^{2+}$ entry into neurones [270] anterior pituitary cells [271] and adrenal chromaffin cells [272] and also stimulate the secretion of [$^3$H]D-aspartate, prolactin and catecholamines from the respective cell types. Certain experiments require caution however, because phytic acid is an extremely potent $Ca^{2+}$ chelator.

There are several phytic acid binding proteins in the nervous system. One of these proteins has the same amino-acid sequence as the 50kDa $\alpha$-subunit of the assembly protein (AP)-2. AP-2 is associated with clathrin-coated vesicles at the plasma membrane but not at the *trans*-face of the Golgi complex and is involved in the regulation of receptor-mediated endocytosis and exocytosis rather than in secretion of newly synthesised proteins. [273-275]

### 2.9.3 *myo*-Inositol Polyphosphate 5-Phosphatase

The *myo*-inositol polyphosphate 5-phosphatase is present in most tissues and found between membranes and cytosol. [58] The 5-phosphatase enzyme removes the 5-phosphate group from several metabolic intermediates including Ins(1,4,5)$P_3$, Ins(1,3,4,5)$P_4$ and *myo*-inositol-1,2-cyclic-4,5-trisphosphate, [Ins(1:2-c,4,5)$P_3$]. The latter compound is hydrolysed by the 5-phosphatase some 100–200 fold more slower than $Ins(1,4,5)P_3$, which may explain the accumulation of cyclic phosphates in cells. [276]

Most tissue 5-phosphatase activity is associated with the particulate fraction [277] however, cells such as platelets may contain large amounts of the soluble form. [259] In brain there are two types of 5-phosphatase, type I and type II, [278,279] defined on the basis of the order of elution from DEAE (diethylaminoethyl) resin. Type I 5-phosphatase has an apparent molecular mass of 45kDa in human platelets. [280] The enzyme requires $Mg^{2+}$ and is inhibited by high concentrations of $Ca^{2+}$. Type I and the particulate 5-phosphatase have similar $K_m$ values for $Ins(1,4,5)P_3$ of about 3μM and 18μM respectively [279] and they share an antigenic determinants. [281] This may suggest that in brain, most 5-phosphatase activity is type I. This enzyme has a higher $K_m$ value for $Ins(1,3,4,5)P_4$ than for $Ins(1,4,5)P_3$ (with a lower $v_{max}$) suggesting that the 5-phosphatase is rapidly saturated with $Ins(1,3,4,5)P_4$, particularly in brain which synthesises more $Ins(1,3,4,5)P_4$ than any other tissue. Consistent with what is already known, 1μM of $Ins(1,3,4,5)P_4$ inhibits the breakdown of either $Ins(1,4,5)P_3$ or $Ins(1{:}2\text{-c},4,5)P_3$ which may prolong the half-lives of these molecules *in vivo*.

Type II soluble 5-phosphatase has a much higher specificity for $Ins(1,4,5)P_3$ ($K_m$ = 24μM) and may be an additional regulatory enzyme. It hydrolyses the same substrates as type I from platelets, although the kinetic constants are different, especially for $Ins(1,3,4,5)P_4$ ($K_m$ = 7.5μM), [282] for type I (range 0.8–1.0μM) [277] and has a molecular mass of 160kDa.

Type I 5-phosphatase from human platelets is activated by PKC phosphorylation. [283] The platelet type II 5-phosphatase is however, not phosphorylated by PKC. [282] The outcome of this difference is a complex regulation of intracellular *myo*-inositol phosphates. One awaits how the enzymes interact *in vivo*, and how *myo*-inositol phosphates are regulated by the complex interelationships between 5-phosphatase and 3-kinase.

### 2.9.4 *myo*-Inositol Polyphosphate 3-Kinase

$Ins(1,4,5)P_3$ may be converted to $Ins(1,3,4,5)P_4$ by a specific 3-kinase, in the presence of ATP and $Mg^{2+}$. [284] The enzyme does not phosphorylate $Ins(1{:}2\text{-c},4,5)P_3$ [285] and has a high affinity for $Ins(1,4,5)P_3$ ($K_m$ 0.2–1.5μM). [286] The 3-kinase competes effectively with the 5-phosphatase for Ins(1,4,5)$P_3$ since the $K_m$ value for the 5-phosphatases is in the range 17–25μM. [280,282] The 3-kinase has been purified from brain [287,288] and its cDNA has been cloned and sequenced. [289,290] The predicted molecular mass (50kDa) is in close agreement to that found by SDS-PAGE. $Ca^{2+}$ increases Ins(1,4,5)$P_3$ 3-kinase activity, an effect which is mediated by calmodulin. [288] The control by intracellular $Ca^{2+}$ levels is also shared by other regulatory enzymes, such as glycogen synthase and cyclic nucleotide phosphodiesterase and represents a mechanism where the ratio of phosphatase to kinase may be modulated. Quantitative analysis has indicated that $Ca^{2+}$/calmodulin binds to the enzyme with a $K_a$ value of 8.23nM (one of the highest affinities for a calmodulin activated enzyme). [291] A rise in $[Ca^{2+}]_i$ thus has an effect of increasing the concentration of Ins(1,3,4,5)$P_4$ first by the action of 3-kinase on Ins(1,4,5)$P_3$ and second by inhibition of Ins(1,4,5)$P_3$ dephosphorylation. The 3-kinase has been shown to be enriched in sequences that contain five amino acids (aspartate, glutamate, proline, serine and threonine) which render the 3-kinase susceptible to hydrolysis by the protease calpain. [286] Regulation of the 3-kinase is also by phosphorylation of the enzyme. It has also been demonstrated that PKA- and PKC-induced phosphorylation of serine residues on brain Ins(1,4,5)$P_3$ 3-kinase increase and decrease, respectively, the $v_{max}$ of enzyme activity.

### 2.10 The Calcium Mobiliser Cyclic ADP Ribose

It is well documented that Ins(1,4,5)$P_3$ releases $Ca^{2+}$ from intracellular $Ca^{2+}$ stores as described in earlier sections. Certain agonists may in addition to producing Ins(1,4,5)$P_3$ also activate a ryanodine sensitive $Ca^{2+}$ channel. [292,293] In pancreatic acinar cells, normal $Ca^{2+}$ mobilisation patterns cannot be mimicked by the action of Ins(1,4,5)$P_3$ alone. However, co-treatment with caffeine gives a similar response as the agonist. [293] Even though caffeine is not the physiological ligand, it has been suggested that a caffeine-like substance activates the ryanodine sensitive $Ca^{2+}$ channel. One such physiological molecule which may be a $Ca^{2+}$ mobilising agent is a metabolite of $NAD^+$ (Nicotinamide Adenide Dinucleotide), namely cyclic ADP-ribose [294] (Figure 13). Cyclic ADP-ribose **(16)** is a $Ca^{2+}$-mobilising agent with a half maximum response of 18nM in purified sea urchin egg microsomes. [295] The cyclic metabolite has a molecular mass of 541Da and is formed by condensing the adenine group of $NAD^+$ with the terminal ribosyl unit and displacing the nicotinamide moiety. [296] The enzyme which forms cyclic ADP-ribose has been found in many tissues including rabbit liver, brain, heart, spleen and kidney [297] and also in pituitary cells. [298] The enzyme, called ADP-ribosyl cyclase has several isoforms and has been purified from various sources. [299,300] The release of $Ca^{2+}$ activated by cyclic ADP-ribose is separate from that of Ins(1,4,5)$P_3$ because it is insensitive to the Ins(1,4,5)$P_3$ receptor antagonist, heparin. [294] Ins(1,4,5)$P_3$ is also less effective than cyclic ADP-ribose at competing for the cyclic ADP-ribose binding site by a factor of more than 1000. [301]

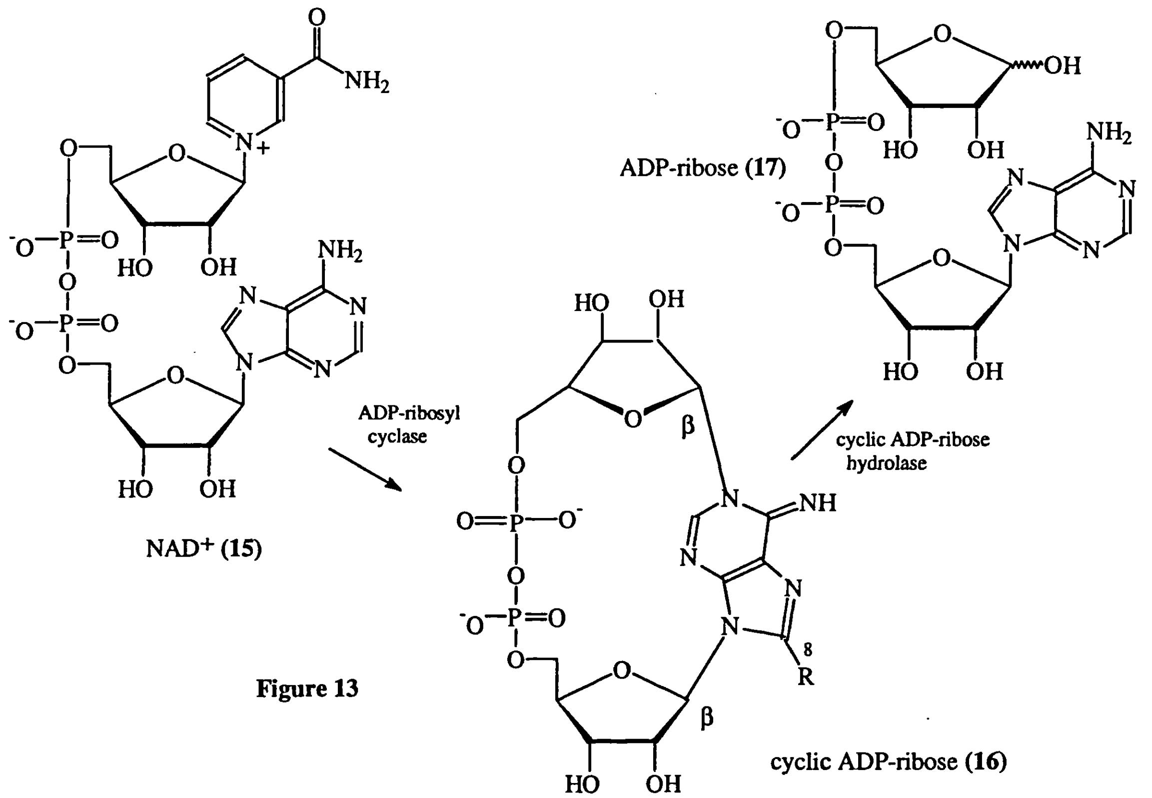



**Figure 13**

Ryanodine opens $Ca^{2+}$ channels at nanomolar concentrations, but closes the channels at higher, micromolar concentrations. The ryanodine receptors contribute to $Ca^{2+}$ signalling in many cells including skeletal muscle, cardiac muscle, neurones, chromaffin cells, smooth muscle, pituitary cells and sea urchin eggs. The mechanism of $Ca^{2+}$-release by cyclic ADP-ribose is similar to another mechanism named CICR. Both ryanodine and caffeine are activators of the CICR mechanism in muscle [302,303] and the two compounds abrogate the response to cyclic ADP-ribose while leaving the $Ca^{2+}$-release mechanism intact. [304] The reverse is also true because egg homogenates become insensitive to caffeine and ryanodine after prior treatment with large concentrations of cyclic ADP-ribose. [304] More recent has been the evidence that in cardiac ryanodine receptors (present in planar lipid bilayers) the frequency of channel opening is increased by cyclic

ADP-ribose. [305] Cyclic ADP-ribose has no effect on type-1 ryanodine receptor of skeletal muscle but is effective in type-2, cardiac receptors [305] and perhaps type-3 receptor. This evidence appears to directly support cyclic ADP-ribose as a second messenger. However, a little caution must be taken because it appears to have no effect in certain cell types. Maybe the cyclic ADP-ribose is more restricted than Ins(1,4,5)$P_3$ in $Ca^{2+}$-release and cell function?

The crucial prerequisite to ascertain that cyclic ADP-ribose is a second messenger, is to show that levels of cyclic ADP-ribose change in response to specific stimuli. This has been observed, albeit controversially, in the levels of cyclic ADP-ribose which change in pancreatic β-cells in response to stimulation with glucose. [306] It has also been shown that cyclic GMP enhanced the formation of cyclic ADP-ribose. [307] There may also be a link with nitric oxide (NO), a local hormone, and it may be fruitful to demonstrate that levels of cyclic ADP-ribose change in response to other types of agonist such as neurotransmitters. The link between cyclic GMP and cyclic ADP-ribose is a crucial step to substantiate that cyclic ADP-ribose is a messenger which regulates some of the ryanodine receptors.

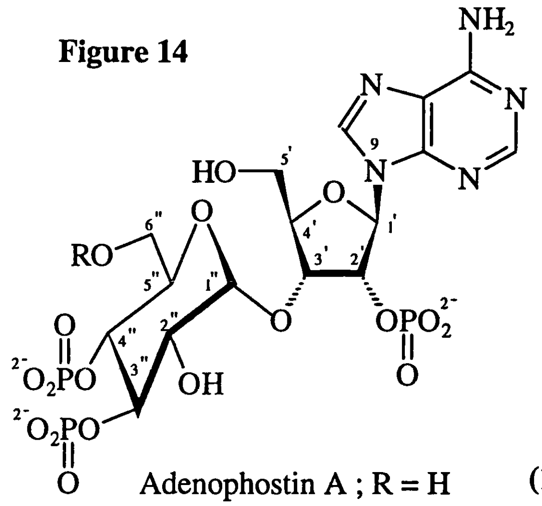
### 2.10.1 Cyclic ADP-Ribose Antagonists

Many cells contain both Ins(1,4,5)$P_3$ receptors and ryanodine receptors and it is known that both compounds contribute to the fertilisation $Ca^{2+}$ wave in sea urchin eggs. [308,309] If one pathway is inhibited, for example the Ins(1,4,5)$P_3$ sensitive $Ca^{2+}$ channel with heparin then the fertilisation response is still intact because the $Ca^{2+}$ wave comes from the back-up mechanism by cyclic ADP-ribose. The cyclic ADP-ribose pathway is inhibited by ruthenium red and, more importantly, by modification of the 8-position of the adenine ring. [308] Substitution of the hydrogen by an amino group at the 8-position of the adenine ring of cyclic ADP-ribose provided an antagonist at 150nM, blocking $Ca^{2+}$-release from sea urchin egg homogenates. The 8-position modified inhibition was reversible and could be overcome by high concentrations of cyclic ADP-ribose. 8-Amino cyclic ADP-ribose was as efficient as cyclic ADP-ribose in binding to its receptor. Finally 8-bromo cyclic ADP-ribose and 8-azido cyclic ADP-ribose were also found to be reversible antagonists, eliminating the $Ca^{2+}$-release but not altering the ability of the molecule to bind to its receptor. [310] In summary, the new $Ca^{2+}$-mobilising molecule, cyclic ADP-ribose, in conjunction with Ins(1,4,5)$P_3$ may generate complex $Ca^{2+}$ signals which may be the key to unlocking the fundamental processes of life.

## 2.11 Adenophostin, the Most Potent Reported Mobiliser of $Ca^{2+}$ at the Ins(1,4,5)$P_3$ Receptor

Previous sections have discussed how an agonist stimulates the production of Ins(1,4,5)$P_3$ *via* enzymatic cleavage of PtdIns(4,5)$P_2$, which then binds to its receptor and releases $Ca^{2+}$ from intracellular stores. Until recently, there were no other naturally occurring small ligands binding to the Ins(1,4,5)$P_3$ receptor and releasing $Ca^{2+}$, or inhibiting the release of $Ca^{2+}$. However, Takahashi and coworkers, [311] have recently described two unique antibiotics, adenophostin A **(18)** and adenophostin B **(19)**, which were isolated from the cultured broth of *Penicillium brevicompactum* SANK 11991 and SANK 12177, respectively (Figure 14). Both adenophostins are potent Ins(1,4,5)$P_3$ receptor agonists and release $Ca^{2+}$ from Ins(1,4,5)$P_3$ sensitive stores. The structures of the adenophostins are unique and have been characterised by UV and NMR experiments, chemical and enzymatic degradation experiments together with elemental analysis and high resolution mass spectra. [312] These techniques have assigned the structure of the adenophostins to a glucose unit possessing phosphate groups on the C-3" and C-4" positions, with a glucosyl linkage at the C-3' position on the ribose sugar of adenosine. There is also a phosphate group on the C-2' ribose unit. [311,312] The only structural difference between adenophostin A and B is that adenophostin B has an acetate group present on the C-6" hydroxyl group of the glucose unit. The chemical structures of adenophostins A and B are distinct from Ins(1,4,5)$P_3$ except for the presence of three phosphate groups. The structures of adenophostin A and Ins(1,4,5)$P_3$ have been modelled using the Alchemy II program. It was found that the three phosphate groups of adenophostin A could not be superimposed on the Ins(1,4,5)$P_3$ structure. [313] However, the 3"- and 4"-diequatorial bisphosphate on the glucose ring could be superimposed on the 4,5-bisphosphate of Ins(1,4,5)$P_3$. It is the 4,5-bisphosphate on Ins(1,4,5)$P_3$ that is important for the release of $Ca^{2+}$, the 1-phosphate being less important. However, the 2'-phosphate on the ribose ring of the adenophostins must be more effective at binding to the receptor than the 1-phosphate of Ins(1,4,5)$P_3$. Removal of the 2'-phosphate on the ribose sugar reduced the binding to the receptor by a factor of 1000.

**Figure 14**



Adenophostin A ; R = H **(18)**

B ; R = $COCH_3$ **(19)**

The adenophostins represent the first microbial (fungal) metabolites isolated which are receptor agonists, and are some 100-fold more potent than Ins(1,4,5)$P_3$ itself. For example, in a competition assay, the binding of adenophostins to rat cerebellar Ins(1,4,5)$P_3$ receptor was found to inhibit [$^3$H]Ins(1,4,5)$P_3$ binding more potently than Ins(1,4,5)$P_3$ itself: the $IC_{50}$ values for adenophostin A, B and Ins(1,4,5)$P_3$ were 1.3, 1.3 and 23nM respectively. The $K_i$ values for adenophostin A and B were both 0.18nM while that of Ins(1,4,5)$P_3$ was 15nM. The adenophostins did not inhibit the binding of [$^3$H]Ins(1,3,4,5)$P_4$ to purified Ins(1,3,4,5)$P_4$ binding protein, demonstrating the high selectivity of these new compounds for the Ins(1,4,5)$P_3$ receptor. Adenophostin A may produce $Ca^{2+}$ release at a concentration as low as 1nM, indeed, after 1min the amount of $Ca^{2+}$ released by 10nM of adenophostin A was equivalent to that released by 1μM of Ins(1,4,5)$P_3$. In addition, whereas Ins(1,4,5)$P_3$ is quickly metabolised, the adenophostins demonstrate sustained $Ca^{2+}$ release. The $EC_{50}$ (the concentration required for half maximum $Ca^{2+}$-release) for adenophostin A, B and Ins(1,4,5)$P_3$ were 1.4, 1.5 and 170μM respectively in cerebellar microsomes. When Ins(1,4,5)$P_3$ was added after sufficient $Ca^{2+}$-release by adenophostin A, no additional $Ca^{2+}$-release was observed indicating that $Ca^{2+}$ is released from Ins(1,4,5)$P_3$ stores. Furthermore, incubation of the microsomes with heparin resulted in the complete disappearance of $Ca^{2+}$-release by adenophostin A, showing that the adenophostins are Ins(1,4,5)$P_3$ receptor agonists. The prolonged activities of the adenophostins to the Ins(1,4,5)$P_3$ metabolising enzymes, 3-kinase and 5-phosphatase have also been investigated. It was found that the binding affinities did not decrease even after a 30min exposure to the enzymes. [313]

In summary, new highly potent $Ca^{2+}$-mobilising compounds called adenophostins, extracted from microbial sources have been discovered. These antibiotics release $Ca^{2+}$ from Ins(1,4,5)$P_3$-sensitive stores and are resistant to the Ins(1,4,5)$P_3$-metabolising enzymes, 3-kinase and 5-phosphatase. The adenophostins induce $Ca^{2+}$-release through the Ins(1,4,5)$P_3$ receptor and has higher activity and specificity than Ins(1,4,5)$P_3$ itself. Adenophostins and structurally modified analogues (to be synthesised) will hopefully be useful tools to further our understanding of the Ins(1,4,5)$P_3$ receptor.

## 2.12 Some Recent Developments in Signal Transduction

New phospholipases C from the β-family have been identified by several groups together with interesting revelations concerning PLC-δ1. First, a new PLC-β was cloned, sequenced and characterised as PLC-β4. [314] PLC-β4 is the most selective PLC for PtdIns(4,5)$P_2$ over PtdIns (at present), and has a molecular mass of 134kDa. PLC-β4 was activated by all the α-subunits [315] of the $G_q$ class including $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$, but none of the G-βγ subunits tested. PLC-β4 shares the highest homology with Norp A protein that mediates the visual process in *Drosophila* [316] suggesting a role for PLC-β4 in the mammalian visual system.

Two other PLC-β types have been identified and characterised from lower eukaryotic sources. First, PLC-*X*β was cloned from *Xenopus* oocytes and found to have substantial homology (33-64%) with mammalian β1, β2, β3 and β4 and was nearest to PLC-β3 with 80% similarity and 64% identity. PLC-*X*β appears to be involved in the pertussis toxin-sensitive receptor stimulation of the $Ca^{2+}$-activated $Cl^-$ current in *Xenopus* oocytes, which only respond to the Gα-subunits.

A second novel form of PLC-β has been found in embryos of the brine shrimp *Artemia*. [317] *Artemia* is of interest from an evolutionary point of view because it branched off considerably earlier than *Drosophila* in the evolutionary tree, with the nearest relative dating back some 550 million years. Therefore the analysis of the PLC sequence between *Artemia* and higher organisms may shed some light on the fundamental structural requirements for PLC function. This PLC type was named PLC-$\beta_x$ and interestingly was activated by concentrations of lithium ions used to treat manic depression.

Another family of phospholipases, named PLC-δ is smaller than the PLC-β or the PLC-γ family. Very little is known about the types of receptor coupled to PLC-δ except that PLC-δ1 is associated with thrombin-triggered mitogenic response, in which PLC-δ1 activation is regulated by G-proteins and calcium. [318] Furthermore, it has been shown that binding of PLC-δ1 to lipid bilayer membranes [composed of phosphatidylcholine and PtdIns(4,5)$P_2$] was inhibited by D-Ins(1,4,5)$P_3$ with an $IC_{50}$ value of 5.4μM. [319] However, L-Ins(1,4,5)$P_3$ did not inhibit the binding process. D-Ins(2,4,5)$P_3$ and 1-(α-glycerophosphoryl)-D-*myo*-inositol 4,5-bisphosphate were nearly as effective. Other analogues such as D-Ins(1,4)$P_2$ weakly inhibited the binding process, but D-Ins(4,5)$P_2$ was nearly as potent as Ins(1,4,5)$P_3$ and PtdIns(4,5)$P_2$. Inorganic phosphate and ATP did not significantly affect binding. Another study [320] has located the D-Ins(1,4,5)$P_3$ binding region on PLC-δ1 to the amino terminal where a peptide relating to residues 30-43 (containing 6 basic amino acids) of PLC-δ1 binds to D-Ins(1,4,5)$P_3$ immobilised on a matrix. Thus, these results are consistent with feedback inhibition by the product of PLC hydrolysis, *i.e.* D-Ins(1,4,5)$P_3$, at a step that preceeds catalysis, namely interfacial stereospecific recognition.

The enzyme PtdIns 3-kinase has received much attention over the last few years. Its structure has been described in section (2.5.2) and the inhibition of this enzyme will be discussed briefly. PtdIns 3-kinase has been implicated in growth factor signal transduction by associating with the tyrosine kinases, including platelet-derived growth factor receptor. Specific inhibitors of PtdIns 3-kinase will hopefully piece together some of the clues to form and function, which are held within the protein architecture of this enzyme. The bioflavinoid quercetin, has been shown to inhibit PtdIns 3-kinase with an $IC_{50}$ value of 3.8μM, [321] but it can also inhibit other kinases such as PtdIns 4-kinase and several tyrosine and serine/threonine kinases. The compound 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one was found to be a selective inhibitor of PtdIns 3-kinase with a potency 2.7-fold greater than quercetin with an $IC_{50}$ value of 1.40μM. [322] The compound did not interact with other kinases tested including PtdIns 4-kinase, EGF receptor tyrosine kinase, MAP kinase and S6-kinase. The fungal metabolite wortmannin has been shown to bind covalently to the ATP binding site of the p110 subunit of PtdIns 3-kinase and inhibit the enzyme irreversibly when added at nanomolar concentrations in mammalian cells. [323] At this low concentration wortmannin does not affect other protein and lipid kinases. However, at micromolar concentrations wortmannin inhibits other enzymes such as phospholipase D [324-326] and myosin light chain kinase. [327] Another compound, demethoxyviridin [328] has also been used as a tool to investigate PtdIns 3-

kinase. The use of these small molecules and other inhibitors will give a greater insight into the functional activity of this enzyme.

In the last few months a paper presented in *Nature* has demonstrated that Ins(1,4,5)$P_3$ inactivates its own specific intracellular $Ca^{2+}$ channel. [329] The inhibitory effect of Ins(1,4,5)$P_3$ can be reversed by Ins(1,4,5)$P_3$ washout or chelation of cysosolic $Ca^{2+}$. Thus, both Ins(1,4,5)$P_3$ and $Ca^{2+}$ act as co-inhibitors of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ channel. The Ins(1,4,5)$P_3$ inactivation may provide a mechanism for adaptation to partially elevated basal levels of Ins(1,4,5)$P_3$. The inactivation should be most potent once the $Ca^{2+}$ channel is open and the $Ca^{2+}$ is released. The process of $Ca^{2+}$ channel inactivation should serve to limit the duration of channel opening and may play an important role in the termination phase of $[Ca^{2+}]_i$ spikes.

In the last few years, several disease states have been attributed to a defect of the *myo*-inositol phospholipid signalling pathway. The first was Lowe's oculocerebrorenal syndrome [330] and second was a defect seen in the phosphatidylinositol pathway in HIV-infected lymphocytes and lymphoblastoid cells. [331] The two disease states share a common cause in that they are both defective in a 5-phosphatase enzyme. Lowe's oculocerebrorenal syndrome has an open reading frame which encodes a protein with 71% similarity and 53% identity to human *myo*-inositol polyphosphatase 5-phosphatase, and thus may be the first identified inborn error of *myo*-inositol phosphate metabolism. This inborn error is a rare X-linked disorder which is characterised by mental retardation and affects the lens opacity, brain, kidneys and muscle. Recently, characterisation of a cDNA encoding the 43kDa membrane-associated *myo*-inositol polyphosphatase 5-phosphatase has been presented. [332] This 43kDa 5-phosphatase was expressed in the heart, skeletal muscle and brain with no significant homology between the 5-phosphatase and other proteins of the phosphoinositide pathway. However, and significantly, a 73-amino acid sequence in the carboxyl terminus of the membrane associated 5-phosphatase enzyme showed a 30% sequence identity and 67% similarity to a region in the 75kDa 5-phosphatase and 34% identity and 70% similarity to a specific sequence in the protein that is encoded by the defective gene in Lowe's oculocerebrorenal syndrome.

Lymphocytes which are infected with HIV *in vivo* showed a defect at the level of the Ins(1,3,4,5)$P_4$ 5-phosphatase together with abnormal $Ca^{2+}$ regulation. It is uncertain at present whether it is the same enzyme that also dephosphorylates Ins(1,4,5)$P_3$ to Ins(1,4)$P_2$. A requirement for tools to investigate the consequences of metabolic dysfunction along the phosphoinositide pathway is demonstrated by the above examples (albeit the mode of inhibition was different) and underpins the importance of pharmacological inhibitors so that we may gain a fruitful understanding of the signal transduction process.

The most outstanding review to date, which covers some of the biological aspects of signal transduction discussed in this review has been presented by M. J. Berridge. [333]

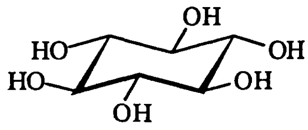
# CHAPTER THREE

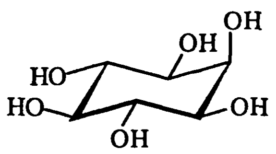
# A Review Of *Myo*-Inositol Phosphate Synthesis

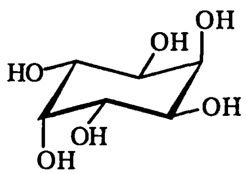
### 3.1 Structure of the Inositols

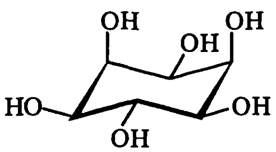
There are nine 1,2,3,4,5,6-hexahydroxycyclohexanes called the inositols. Figure 15 shows all nine possible configurations and their trivial names. Only two of these are optically active: D-**(27)** and L-*chiro*-inositol **(28)**. The other seven isomers are optically inactive, *meso*-cyclitols, with a plane of symmetry through the cyclitol.

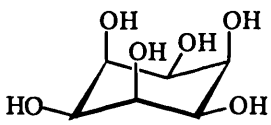
*myo*-Inositol was first isolated by Scherer in 1850 [334] and was given the name "inosit" and later the suffix *ol* in English. *myo*-Inositol is the most common inositol, occuring naturally and appearing in several forms in all living organisms. [335] *myo*-Inositol is extracted from corn-steep liquor in large quantities and sold by suppliers such as Aldrich as a high purity white crystalline solid, at a cost of £29.80, for 500g (Aldrich 1994-1995).

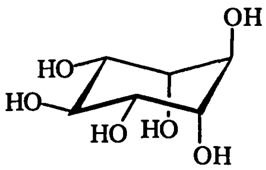

*scyllo*-inositol **(20)**

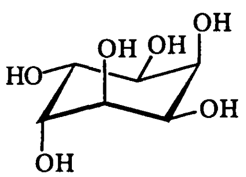

*myo*-inositol **(21)**

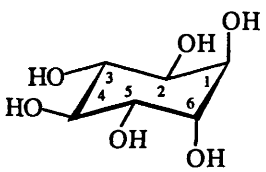

*neo*-inositol **(22)**


*epi*-inositol **(23)**


*cis*-inositol **(24)**


*muco*-inositol **(25)**


*allo*-inositol **(26)**


D-*chiro*-inositol **(27)**

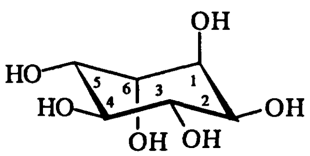
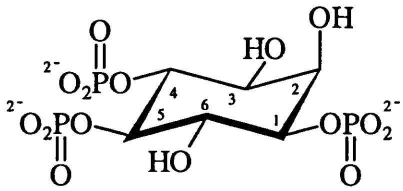
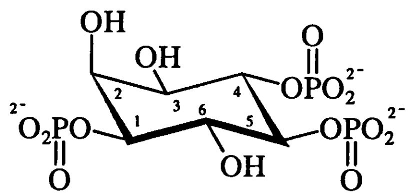

L-*chiro*-inositol **(28)**

**Figure 15**

*myo*-Inositol possesses a single axial hydroxyl group, and the carbon to which it is attached is designated position 2. The C-1 position of *myo*-inositol is designated to the carbon nearest position 2. Asymetric substitution of the *myo*-inositol configuration is, by convention, L when the numbering proceeds clockwise from 1→6, and is D when the numbering proceeds anticlockwise. The choice of prefix is normally determined by giving preference to that which results in the lowest numbering of substituents and the two configurations are shown in Figure 16 for D-**(7)** and L-*myo*-inositol 1,4,5-trisphosphate **(29)**. In a metabolic sequence however, for example in the dephosphorylation of D-Ins(1,3,4)$P_3$ to give D-Ins(3,4)$P_2$ the lowest nomenclature should be L-Ins(1,6)$P_2$, but recent recommendations [336] on the numbering of the atoms of *myo*-inositol relax this rule and the D-prefix is used in order to avoid confusion.



D-Ins(1,4,5)$P_3$ **(7)**



L-Ins(1,4,5)$P_3$ **(29)**

**Figure 16**

The rules for cyclitol nomenclature are often confusing and long. [337] However, two main points of note are: first, when numbering substituted carbons, the substituents must be placed in alphabetical order, for example, allyl before benzyl which comes before prop-1-enyl, regardless of the position of the substituent in the ring. Second, a small Roman capital letter should be used in print for D- and L- derivatives and *sn* is an abbreviation for stereospecific numbering.

### 3.2 The Biosynthesis of *myo*-Inositol

Most *myo*-inositol for cellular processes in humans comes from dietary intake especially from vegetation. A small proportion of *myo*-inositol is biosynthesised. *De novo* biosynthesis occurs in man by isomerisation of D-glucose-6-phosphate **(30)** to give L-*myo*-inositol-1-phosphate **(31)** in a NAD-dependent process requiring the enzyme L-*myo*-inositol synthase. This enzyme is present in all animals and plants and is abundant in the mammalian testis, from which source it has been purified, [338,339] and also occurs in brain (*vide infra*).

The reaction mechanism includes a stereospecific intramolecular aldol condensation [340] *via* a keto intermediate, to give the aldol product, L-*myo*-2-inosose-1-phosphate. The ketone is then stereospecifically reduced by NADH to give L-*myo*-inositol-1-

phosphate followed by dephosphorylation by *myo*-inositol-1-phosphatase to give *myo*-inositol (Figure 17).

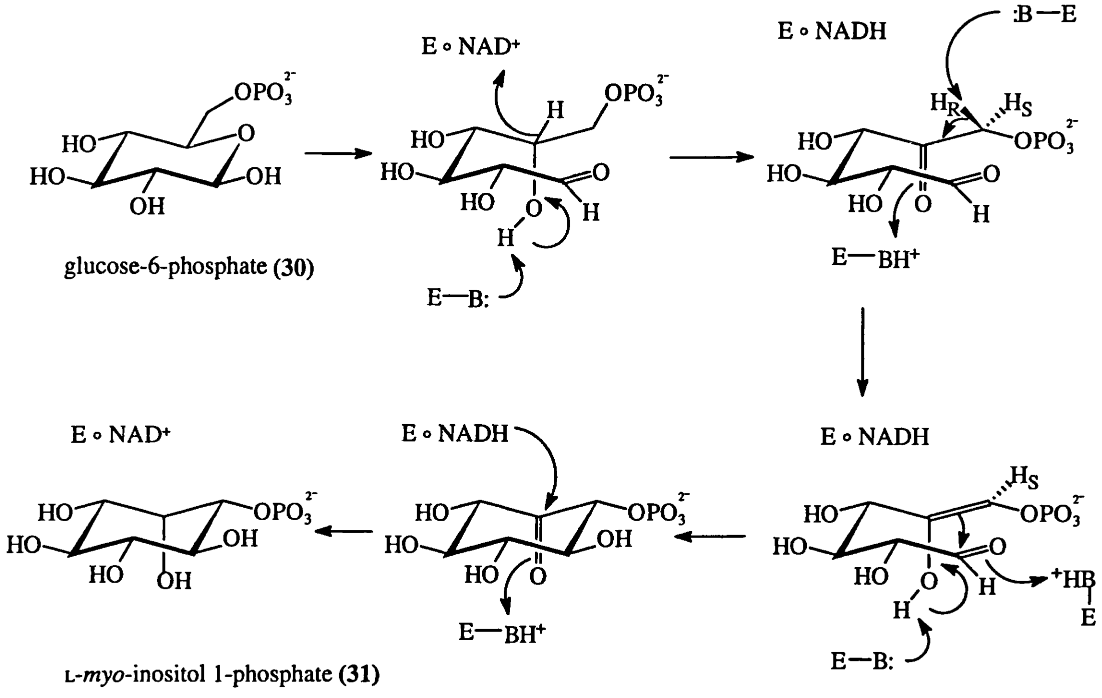



**Figure 17**

### 3.3 Introduction to the Synthesis of *myo*-Inositol Phosphates

The following sections of this chapter will deal with the principals for the synthesis of inositol phosphates and analogues. The preparation of these compounds poses four distinct problems, all of which have been solved. First, using *myo*-inositol as starting material, suitable protecting groups must be introduced into the *myo*-inositol ring so that phosphates (or other groups) may be incorporated into their respective positions. Second, *myo*-inositol is a *meso* compound and the natural configuration of Ins(1,4,5)$P_3$ is the D-enantiomer. It is therefore necessary to resolve racemic *myo*-inositol phosphate precursors. Third, an efficient phosphorylation methodology must be employed with a reagent possessing suitable phosphate-protecting groups in order to avoid the formation of cyclic phosphates and also to effect efficient phosphorylation of the vicinal diol so inositol polyphosphates may be successfully synthesised. Finally, once protected phosphates (or other similar functional groups) are on the ring, the protecting groups must be cleanly removed without causing migration or cyclisation of the phosphates.

## 3.4 The Use of Protective Groups in the Synthesis of *myo*-Inositol Phosphate Precursors

### 3.4.1 Protection of Diols with Acetal Groups

*myo*-Inositol is a cheap readily available starting material. Three isomeric bisacetals are produced when cyclohexanone [341] or more appropriately a cyclohexanone precursor [342,343] is reacted with *myo*-inositol in the presence of an acid catalyst. The three isomeric bisacetals (Figure 18) were obtained in pure form by a combination of crystallisation and chromatography. DL-1,2:4,5-Di-*O*-cyclohexylidene-*myo*-inositol **(32)** was recovered from the mixture by crystallisation and the other two isomers, DL-1,2:3,4-**(33)** and DL-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol **(34)** may be separated by chromatography. DL-1,2-*O*-Cyclohexylidene-*myo*-inositol **(35)** was obtained by mild acid hydrolysis of the less stable *trans*-acetal. The di-*O*-cyclohexylidene-*myo*-inositol derivatives can be selectively alkylated or acylated and further manipulated to provide a series of phosphorylation precursors which will be described in due course.

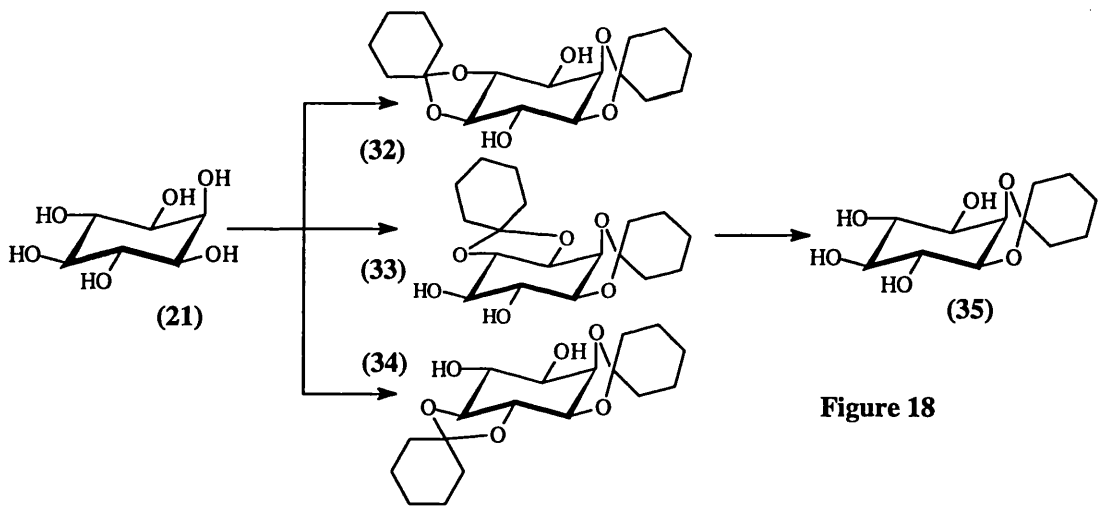
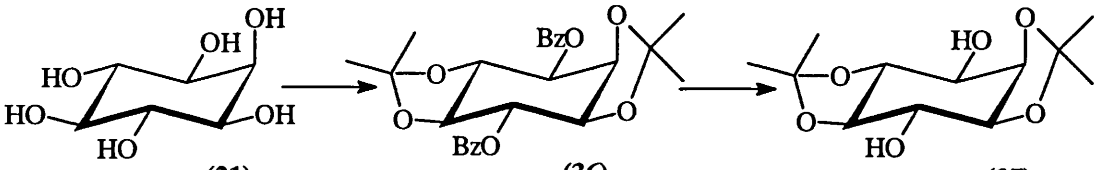



**Figure 18**

The isopropylidene group [344] has also been used to protect *cis*- and *trans*-1,2-diols. It was introduced into the *myo*-inositol ring using 2,2-dimethoxypropane or 2-methoxypropene and an acid catalyst. Initially, derivatisation with benzoyl chloride was necessary in order to obtain DL-3,6-di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(36)**. Since the other benzoylated derivatives were soluble, DL-3,6-di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol was isolated from the mixture by washing with pyridine, ether, water and acetone. The benzoyl esters were then hydrolysed to provide the versatile diol, DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol, **(37)** (Figure 19).



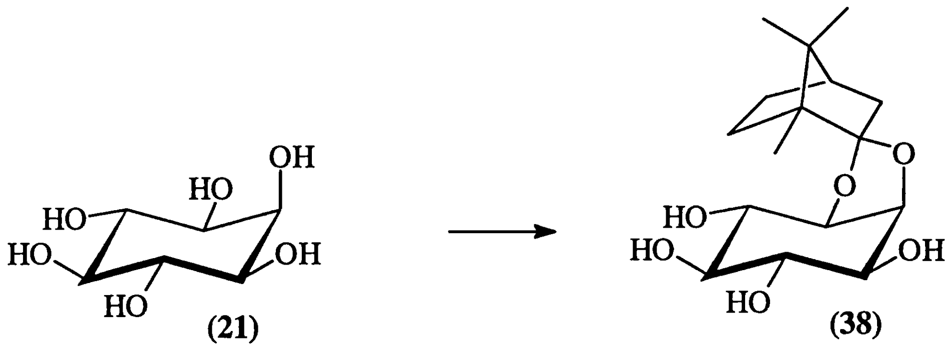
(21) (36) (37)

**Figure 19**

Several years after the above procedure was published, further investigation improved the preparation of isopropylidene acetals of *myo*-inositol. [345] When *myo*-inositol was treated with 5 equivalents of 2-methoxypropene and toluene-*p*-sulphonic acid in dimethyl sulphoxide (DMSO) a mixture of DL-1,2:4,5- DL-1,2:5,6- and DL-1,2:3,4-di-*O*-isopropylidene-*myo*-inositol (in a ratio of 3.5:2:1) was obtained, together with 1,2:3,4:5,6-tri-*O*-isopropylidene-*myo*-inositol in a total yield of 90%. Column chromatography of the mixture gave the tri-*O*-isopropylidene-*myo*-inositol derivative (11%), whereas the DL-1,2:4,5- and DL-1,2:5,6- derivatives were isolated by fractional crystallisation and characterised as the diacetates. Under slightly different conditions, using a shorter reaction time and a lower amount of reagent in the same solvent, a larger proportion of the DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol derivative was produced and the tri-*O*-isopropylidene was not formed. However, changing the solvent from DMSO to DMF increased the formation of DL-1,2:5,6-di-*O*-isopropylidene-*myo*-inositol and gave the compounds, DL-1,2:4,5-, DL-1,2:5,6- and DL-1,2:3,4-di-*O*-isopropylidene-*myo*-inositol in the ratio of 2.5:2:1 in a total yield of 92%.

The cyclopentylidene acetal has also been used. [346] The only advantage of this diol protecting group is that it is more easily removed by acid hydrolysis than cyclohexylidene or isopropylidene.

In a one pot reaction, the treatment of *myo*-inositol and the dimethyl acetal of D-camphor in DMSO and a catalytic amount of acid gave a mixture of acetals which were subsequently hydrolysed to give four possible *cis*-monoacetals. Furthermore, toluene-*p*-sulphonic acid catalysed equilibration of the crude mixture and afforded just one monoacetal, the favoured D-2,3-*O*-camphor-*myo*-inositol **(38)**, (Figure 20) derivative, which precipitated from the mixture in 65% yield and high optical purity. [347] L-Camphor dimethylacetal gave the D-1,2-*O*-camphor-*myo*-inositol derivative. This one pot procedure has been used to protect the *cis*-1,2- and 2,3-diol using the L- and D-camphor derivatives respectively as well as resolving *myo*-inositol with the chiral acetal. The camphor acetal has been further manipulated to provide optically active *myo*-inositol mono- and polyphosphates. [348]



(21) (38)

**Figure 20**

The removal of these 1,2-diol protecting groups may be carried out easily and selectively depending on the acidic conditions. For example, keeping a solution of DL-3,6-di-*O*-benzyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol in 80% acetic acid at reflux temperature hydrolysed both *cis*- and *trans*-diol protecting acetals [344] to give DL-1,4-di-*O*-benzyl-*myo*-inositol. The isopropylidene group was hydrolysed from DL-4,5-di-*O*-allyl-1,2-*O*-isopropylidene-5,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol using 1M HCl –methanol (9:1) at 50°C to expose the *cis*-1,2-diol. [349] A prolonged reaction time hydrolysed the *p*-methoxybenzyl group. The *trans* cyclohexylidene acetal was removed from DL-5,6-di-*O*-benzyl-1,2:3,4-di-*O*-cyclohexylidene-*myo*-inositol using one equivalent of ethane 1,2-diol and a catalytic amount of toluene-*p*-sulphonic acid in chlorinated solvent at room temperature, to expose the *trans*-diol in a 60% yield. [350]

### 3.4.2 The Synthesis and Manipulation of *myo*-Inositol Orthoformate

The reaction of *myo*-inositol with triethylorthoformate at 100–140°C and an acid catalyst afforded a single orthoformate derivative [351] (Figure 21). The reaction was originally carried out using dry DMSO as a solvent in 76% yield. However, DMF replaced DMSO as the solvent of choice providing the orthoformate derivative in a higher yield (92%) and resulting in a cleaner product. [352] The orthoformate structure **(39)** is unique in *myo*-inositol protection chemistry, because the 1-, 3- and 5-positions are protected in one step and the positions of the hydroxyl groups are reversed, so only the 2-hydroxyl is now equatorial and the others are axial. The 1,3-diaxial orientation of the hydroxyl groups in the 4- and 6-positions of the *myo*-inositol ring allowed the regioselective mono-alkylation at one of the axial positions. Selectivity is thought to be initiated by the internal co-ordination of the intermediate alkoxide ion (between the 4- and 6-positions). [353] Monoalkylation occurred in DMF when one equivalent of alkylating reagent such as allyl bromide or benzyl bromide was used together with one equivalent of sodium hydride as base. [353] The yields for this selective reaction are consistent and within the range of 67–80%. For example, for *p*-methoxybenzyl chloride the yield is 67%, for benzyl bromide, 75% and for allyl bromide 80%. Furthermore, treatment of DL-4-*O*-benzyl-*myo*-inositol-orthoformate with sodium hydride (one equivalent) in DMF followed by the addition of benzyl bromide gave *ca.* 5:1 mixture of 4,6- and DL-2,4-di-*O*-benzyl-*myo*-inositol-orthoformate derivatives together with 2,4,6-tri-*O*-benzyl-*myo*-inositol-orthoformate. Direct di-*O*-benzylation of *myo*-inositol-orthoformate in DMF with sodium hydride and benzyl bromide (two equivalents each) gave a mixture of 4,6- and DL-2,4-di-*O*-benzyl derivatives, albeit in a lower yield.

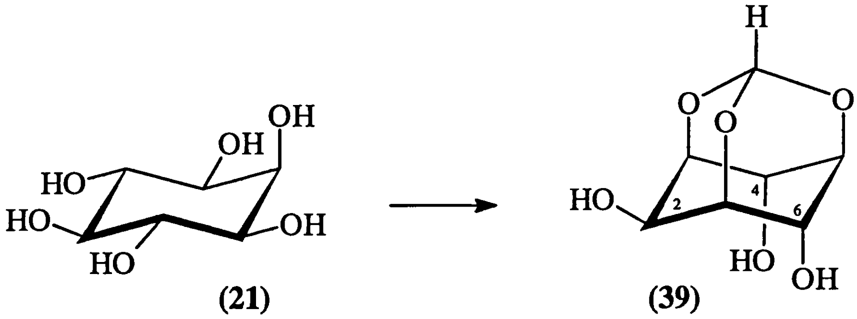



**Figure 21**

### 3.4.3 Other Uses of the Orthoformate Structure

Finally, the orthoformate structure has been utilised to produce analogues of enterobactin, [354] a siderophore produced by enteric bacteria to trap ferric ions [355] under iron-deficient conditions. The monoorthoformate of *scyllo*-inositol **(40)** (Figure 22) was prepared by the procedure described previously. [351] Kishi was particularly intrigued with the three axially disposed hydroxyl groups present at the 1-, 3- and 5-positions in the cyclohexane ring and this structural feature was utilised for the design of enterobactin analogues. Two types of analogues have been synthesised from this *scyllo*-orthoformate derivative. First, a chiral ligand has been attached to each of the three axial hydroxyl groups and the compound may form a complex with chirality at the metal centre. Second, the orthoformate group of the *scyllo*-derivative may be replaced by other longer chain alkyl groups, increasing the lipophilicity and thus tuning the polarity of the metal complexes. Thus, the treatment of the *scyllo*-inositol orthoformate with 3-bromo-2-phenyl-1-propene in the presence of sodium hydride, followed by ozonolysis, gave the triketone **(41)** (77% yield). Asymmetric reduction of the triketone with Corey's (*S*)-oxazaborolidine [(*S*)-CBS] reagent gave the (*S*)-triol, **(42)** with very high stereoselectivity. Mesylation of the triol followed by treatment with tetrabutylammonium azide gave the (*R*)-triazide **(43)** in 64% yield from the triol.

Hydrogenation of the triazide and coupling with $O$-dibenzyl 2,3-dihydroxybenzoic acid followed by catalytic hydrogenation furnished the chiral enterobactin analogue **(44)**.

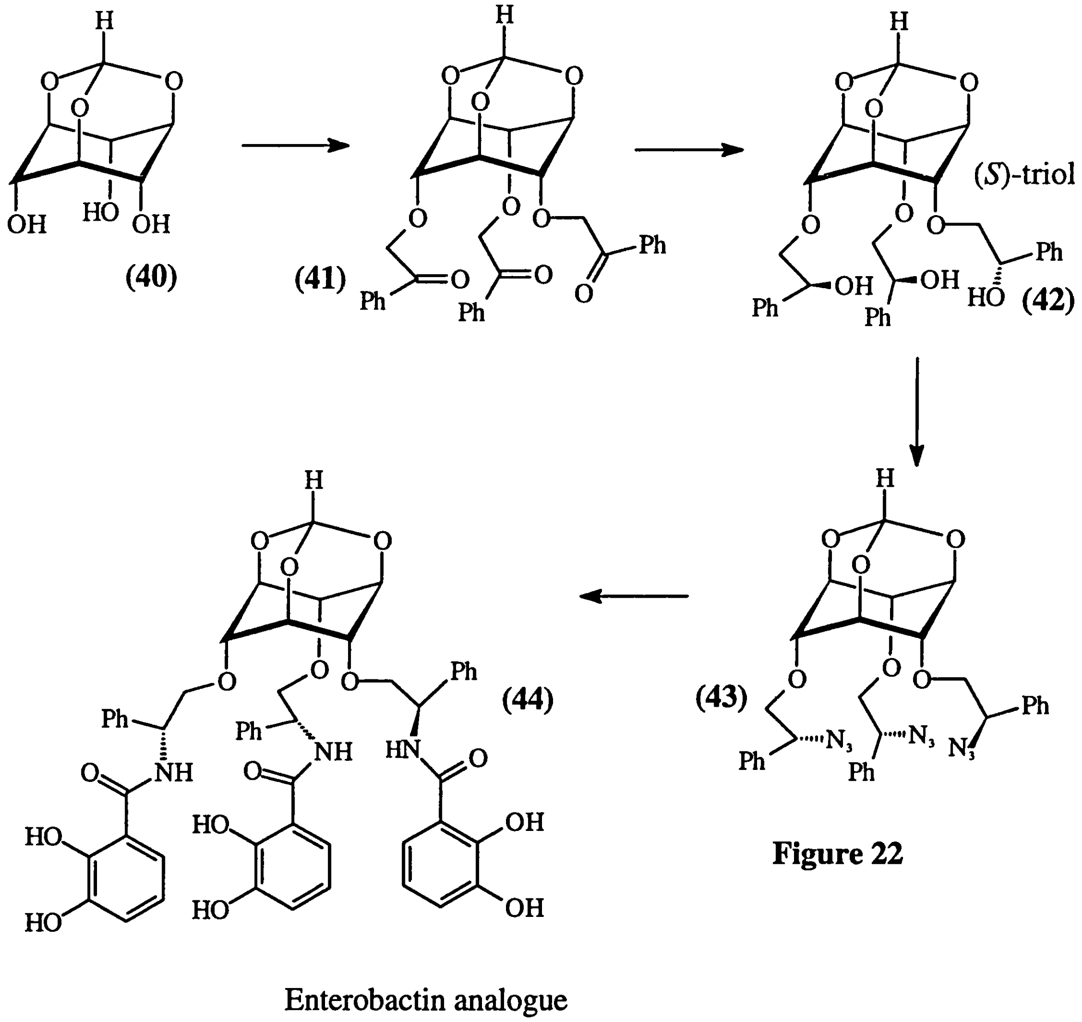



**Figure 22**

The orthoester derivative was replaced by a long alkyl chain, undecyl, $C_{11}$-derivative. Thus hydrolysis of 1,3,5-tri-$O$-benzyl-*scyllo*-inositol monoorthoformate gave 1,3,5-tri-$O$-benzyl-*scyllo*-inositol **(45)**. Reaction of the *scyllo*-derivative with trimethyl orthoundecanoate in the presence of a catalytic amount of toluene-*p*-sulphonic acid followed by catalytic hydrogenation gave the orthoundecanoate **(46)**. This undecyl derivative was manipulated as for the orthoformate to give the more lipophilic analogue **(47)** (in Figure 23). Both analogues chelated ferric ions to give a deep red complex. However, the lipophilic analogue also showed excellent solubility in organic solvents and upon partitioning between water and ethyl acetate, the lipophilic analogue complex only appeared in the organic layer, whereas the monoorthoformate derivative appeared in the aqueous layer. Thus the monoorthoformate derivative may be tuned to alter the chemical properties of the metal complexes.

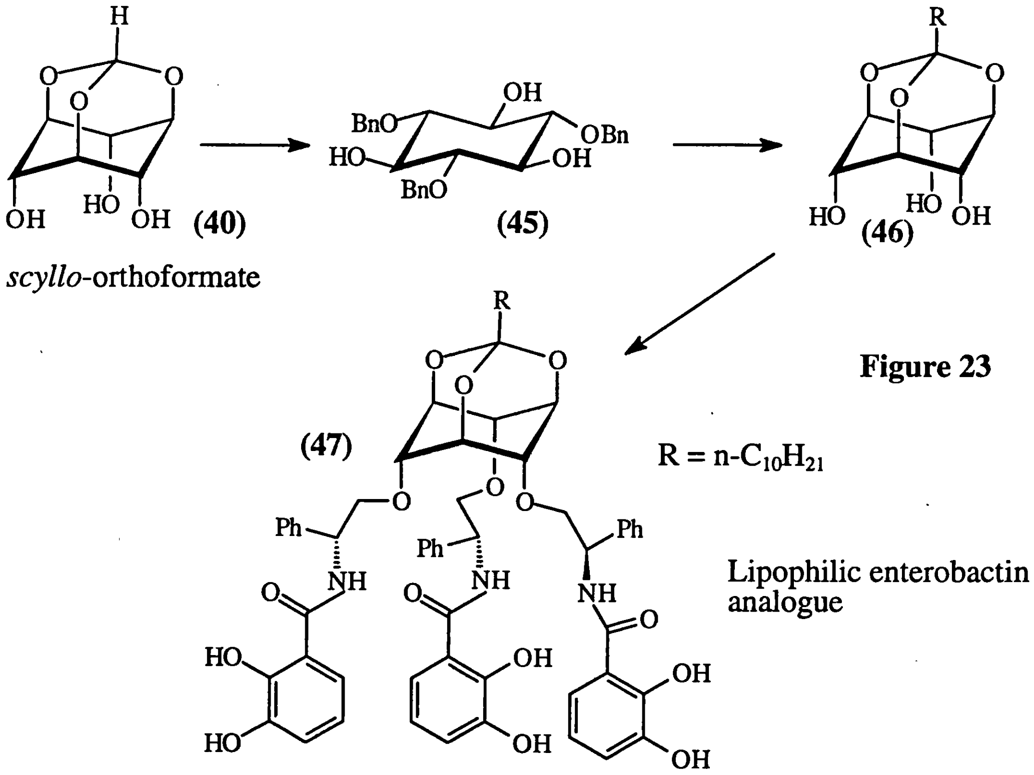



**Figure 23**

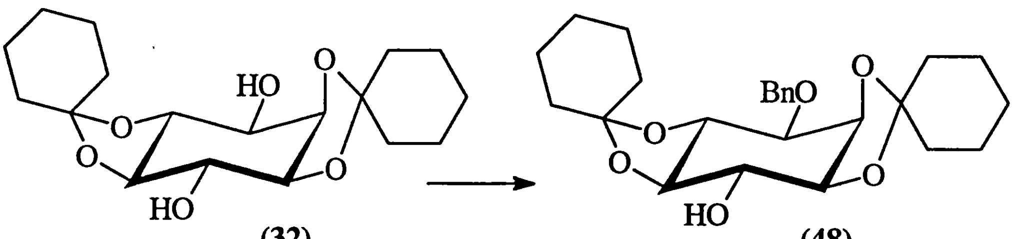
### 3.5 Protection of Hydroxyl Groups with Allyl Ethers

A brief overview of the common hydroxyl protecting groups and their use in the synthesis of *myo*-inositol phosphate precursors will follow. The allyl group is normally introduced into the *myo*-inositol ring using allyl bromide in DMF with sodium hydride as base, at room temperature. [349] Allyl ethers are stable to many conditions used to remove other protective groups, for example, 1M HCl, reflux 10h, [356] which can remove isopropylidene and *p*-methoxybenzyl groups. The deprotection of the allyl group usually takes place in a two-step procedure consisting of rearrangement to a prop-1-enyl ether and may be carried out in several ways. First, isomerisation may be induced by using freshly sublimed potassium *t*-butoxide (five equivalents per allyl) in dry DMSO at 50°C for 3h to give the *cis*-prop-1-enyl ether in near quantitive yield. [357] Second, Wilkinson's catalyst, tris(triphenylphosphine)rhodium (I) chloride [$(PPh_3)_3RhCl$] in the presence of DABCO (1,4-diazabicyclo[2.2.2]octane) with ethanol-toluene-water, (7:3:1, v/v/v) at reflux, gave a mixture of *cis*- and *trans*-prop-1-enyl ethers in a ratio of *ca.* (5:1) and 82% yield. [358] Third, another organometallic reagent, 1,5-cyclooctadiene-*bis*(methyldiphenylphosphine)iridium hexafluorophosphate, used in a catalytic amount, under an inert atmosphere of helium and activated by passing over a stream of hydrogen for 2min also isomerised an allyl group to give a *trans*-prop-1-enyl ether in near quantitative yield. [359]

The resulting prop-1-enyl ethers may be cleaved under several conditions, including 1M HCl-methanol (1:5), reflux 30min in near quantitative yield (95%). [357] Stirring with mercury (II) chloride and mercury (II) oxide (one equivalent of each) in acetone-water (10:1) for 5min furnished the exposed hydroxyl group in 87% yield. [357] A final method for regenerating the hydroxyl group directly, in a two step one pot reaction, uses palladium on carbon with an ethanol-water mixture at reflux and a catalytic amount of toluene-*p*-sulphonic acid. [360] Caution must be taken however, because prolonged reflux leads to loss of benzyl groups and careful monitoring of the reaction mixture is essential. Other methods for allyl ether deprotection may be found in a recent publication. [361] Selective methods for introducing the allyl group into the *myo*-inositol ring will be discussed in section 3.9.1 and 3.9.2 for the first two synthesis of Ins(1,4,5)$P_3$.

### 3.5.1 Benzyl and *p*-Methoxybenzyl Protective Groups

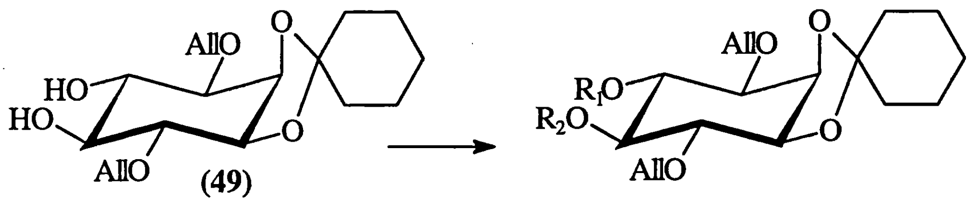
Unsubstituted benzyl ethers were originally prepared under harsh conditions, using the alcohol and neat benzyl chloride in the presence of excess potassium hydroxide for several hours at 130–140°C. [362] Nowadays, however, there are two main methods for introducing benzyl groups into the *myo*-inositol ring. First, DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol was benzylated using benzyl bromide in the presence of sodium hydride as a base with DMF as solvent. The yield was high, 90–95%, after crystallisation from hot petroleum-ether, 60–80°C. The benzylation produced DL-3,6-di-*O*-benzyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol, an important intermediate in the synthesis of DL-1,2,4-tri-*O*-benzyl-*myo*-inositol, [349] which was used to synthesise Ins(1,4,5)$P_3$. A second method involves the mild acid-catalysed reaction of benzyl trichloroacetamidate with the hydroxyl group, under conditions which do not affect the acid sensitive acetal protective groups. Thus, DL-3-*O*-benzoyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol was benzylated at the 6-position in 85% yield with benzyl trichloroacetamidate in dichloromethane and a catalytic amount of trifluoromethane sulphonic acid. [363] The use of strong base such as sodium hydride was not satisfactory in the presence of the benzoyl group. However, sodium hydride and benzyl bromide were used to benzylate the 2-hydroxyl group of DL-2,3,6-tri-*O*-benzyl-4,5-di-*O*-butanoyl-1-*O*-*p*-methoxybenzyl-*myo*-inositol in 89% yield after crystallisation and without hydrolysis of the butanoyl groups. [358]



(32) (48)

**Figure 24**

There are two examples of regiospecific benzylation of DL-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol at the free 3-hydroxyl group, (Figure 24). First, sodium hydride and benzyl bromide (1.1 equivalents each) were added to a solution of the diol with toluene as solvent. The mixture was heated at reflux for 6h then stirred at room temperature overnight. The major product, DL-3-*O*-benzyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol **(48)** could be separated from the DL-3,6-di-*O*-benzyl and DL-6-*O*-benzyl derivatives in 60% yield. [364] In a second example, the synthesis of DL-3-*O*-benzyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol was carried out using 2 equivalents of barium oxide and 0.125 equivalents of barium hydroxide octahydrate in DMF and 1.1 equivalents of benzyl bromide. [365] A final example of regioselective benzylation has appeared in the last few years. The diol, DL-3,6-di-*O*-allyl-1,2-*O*-cyclohexylidene-*myo*-inositol was regioselectively benzylated **(49**; Figure 25) under phase-transfer conditions (tetrabutylammonium hydrogen sulphate in 5% aqueous sodium hydroxide) to afford a 3:2 mixture [4-*O*-benzyl, **(50)** 53%, 5-*O*-benzyl **(51)** 36%] of mono-*O*-benzyl derivatives at the 4- and 5- positions respectively together with a small portion of the di-*O*-benzyl derivative **(52)** (5%). [359] Other selective alkylating methods will be discussed in section 3.6.



(49)

**Figure 25**

$R_1$ = Bn, $R_2$ = H **(50)**
$R_1$ = H, $R_2$ = Bn **(51)**
$R_1$ = Bn, $R_2$ = Bn **(52)**

The benzyl group is resistant to harsh acidic and basic conditions, therefore the majority of the phosphorylation precursors for the synthesis of *myo*-inositol tris- and tetrakis-phosphates in the literature contain di- or tri-*O*-benzyl-*myo*-inositol precursors. The main reason for using benzyl groups is that they can be removed under the same conditions as the benzyl phosphate protecting groups, without migration or cyclisation of the phosphates. The two main methods used for deblocking the protected phosphate are catalytic or chemical reduction. For example, catalytic reduction of D-2,5,6-tri-*O*-benzyl-1,3,4-tris(dibenzyloxyphospho)-*myo*-inositol with 5% palladium on carbon using ethanol as solvent, under an atmosphere of hydrogen, at room temperature for 24h, gave Ins(1,3,4)$P_3$. [350] The second method used to expose the naked *myo*-inositol phosphate is chemical reduction, where the cyanoethoxyphosphate/phosphorothioate protective groups were removed within 15min using sodium in liquid ammonia. [358] Other methods used for the deprotection of benzyl groups may be found in "Protective Groups in Organic Synthesis". [366]

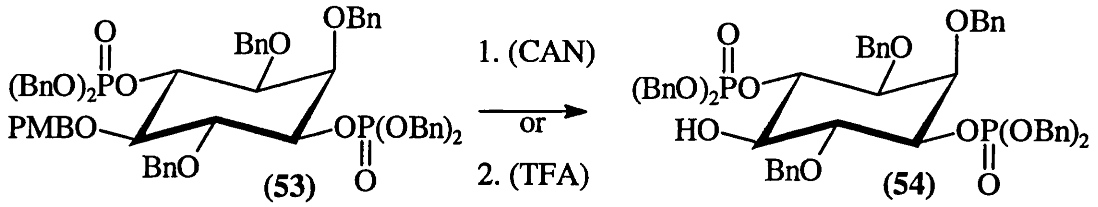


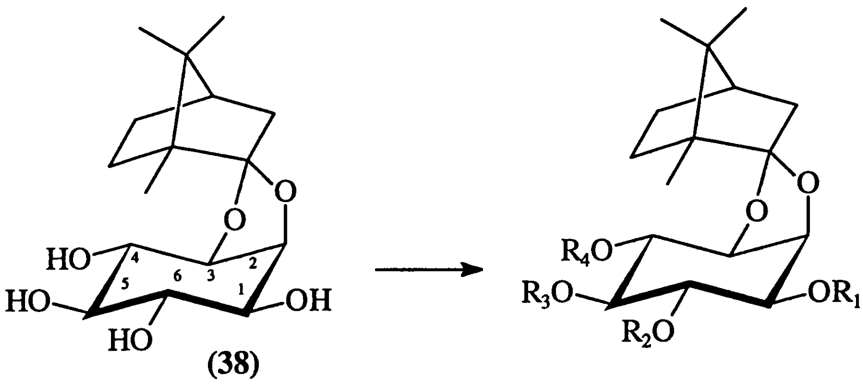
**Figure 26**

The *p*-methoxybenzyl group is not as stable to the harsh acidic conditions as unsubstituted benzyl groups. It is introduced into the *myo*-inositol ring using *p*-methoxybenzyl chloride with sodium hydride as base, in DMF. Its special place in the synthesis of *myo*-inositol phosphate derivatives arises because it may be deprotected by several different methods. First, the *p*-methoxybenzyl group of DL-2,3,6-tri-*O*-benzyl-4,5-bis[di(2-cyanoethoxyphospho)]-1-*O*-*p*-methoxybenzyl-*myo*-inositol, was removed using 1.5 equivalents of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, (DDQ) in dichloromethane-water (15:1) within 1h at room temperature. [358] The *p*-methoxybenzyl group has also been removed from another phosphorylated precursor using cerium (IV) ammonium nitrate. Thus, D-2,3,6-tri-*O*-benzyl-1,4-bis(dibenzyloxyphospho)-5-*O*-*p*-methoxybenzyl-*myo*-inositol (**53** in Figure 26) was stirred at 20°C and treated with a solution of cerium (IV) ammonium nitrate (CAN) in acetonotrile-water (9:1). The product was extracted and purified to give the 5-hydroxyl derivative in 72% yield. [367] Two methods have been used to remove the *p*-methoxybenzyl group under acidic conditions. First, DL-1,2:4,5-di-*O*-isopropylidene-3-*O*-*p*-methoxybenzyl-6-*O*-methyl-*myo*-inositol was heated under reflux in 1M HCl-ethanol (1:2) for 3h to remove the isopropylidene acetals and the *p*-methoxybenzyl group to give DL-4-*O*-methyl-*myo*-inositol. [349] Second, DL-2,3,6-tri-*O*-benzyl-1,4-bis(dibenzyloxyphospho)-5-*O*-*p*-methoxybenzyl-*myo*-inositol **(53)** was treated with 2.5% trifluoroacetic acid (TFA) in dichloromethane. The mixture was stirred for

30min at 20°C and concentrated *in vacuo*, neutralised and chromatographed to expose the 5-hydroxyl group **(54)** in 82% yield. [359]

### 3.5.2 Ester Protective Groups

Esters are generally prepared with an acid chloride or anhydride in the presence of an alcohol using pyridine or triethylamine as a base. The benzoate group has previously been used for derivatising a mixture of di-*O*-isopropylidene-*myo*-inositols to give DL-3,6-di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol. [344] Pivaloyl chloride has been used to derivatise selectively, D-2,3-*O*-camphor-*myo*-inositol. Treatment of D-2,3-*O*-camphor-*myo*-inositol with 1.1 equivalents of pivaloyl chloride in pyridine gave the D-2,3-*O*-camphor-1-*O*-pivaloyl-*myo*-inositol **(55)** derivative in 44% yield. [348] Furthermore, when the tetrol was treated with 2.2 equivalents of pivaloyl chloride D-2,3-*O*-camphor-1,5-di-*O*-pivaloyl-*myo*-inositol **(56)** is formed in 40% yield. Finally, treatment of the same tetrol with 4 equivalents of pivaloyl chloride at room temperature resulted in the prevalent formation of the D-1,4,5-tri-*O*-pivaloyl derivative **(57)** which may be separated from other isomers by column chromatography (Figure 27). These three compounds have been used to synthesise *myo*-inositol phosphates by further derivatisation followed by phosphorylation.




**(38)**

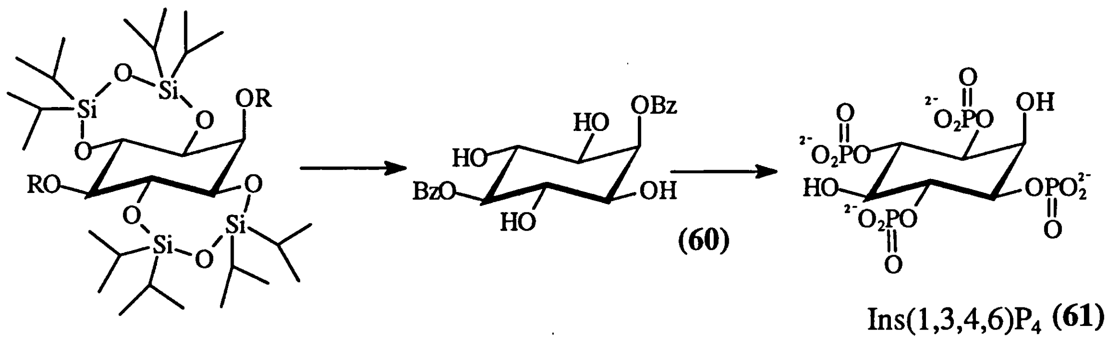
**Figure 27**

$R_1$ = Pivaloyl, $R_2 = R_3 = R_4$ = H **(55)**
$R_1 = R_3$ = Pivaloyl, $R_2 = R_4$ = H **(56)**
$R_1 = R_3 = R_4$ = Pivaloyl, $R_2$ = H **(57)**

*myo*-Inositol has been benzoylated selectively using 3.5 equivalents of benzoyl chloride in pyridine for 2h, to give three main products. [368] The ratio of the benzoylated products was temperature dependent and at 90°C, DL-1,3,4,5-tetra-*O*-benzoyl-*myo*-inositol, 33% was the main product together with 1,3,4,6-tetra-*O*-benzoyl-*myo*-inositol, 13% and DL-1,3,4,5,6-penta-*O*-benzoyl-*myo*-inositol. The DL-1,3,4,5-tetra-*O*-benzoyl-*myo*-inositol derivative was benzylated by the reaction with benzyl trichloroacetimidate and trifluoromethane sulphonic acid followed by debenzoylation with sodium methoxide to give the 1,3,4,5-tetrol. The tetrol was phosphorylated and deprotected to give Ins(1,3,4,5)$P_4$. Ozaki and coworkers have also used the benzoylating reagent benzoyl imidazole [363] in the presence of caesium fluoride (CsF), to benzoylate selectively, DL-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol at the 3-hydroxyl position in 65% yield and with no other regioisomer. The use of other benzoylating reagents gave mixtures of products, so benzoyl imidazole is a useful selective benzoylating reagent. Acetate and butyrate derivatives have not been used widely, but their use in *myo*-inositol chemistry will be described under "Enzymatic Resolutions".

If the benzoylation of *myo*-inositol is carried out using 2.5 equivalents of benzoyl chloride in pyridine at 90°C, 1,3,5-tri-*O*-benzoyl-*myo*-inositol is readily isolated in 15% yield by column chromatography. [369] This intermediate was enantioselectively acylated under kinetic conditions using a tartaric acid monoester to provide a D- or L-tetra-acylated product which was transformed into D-Ins(1,3,4,5)$P_4$ or L-Ins(1,3,4,5)$P_4$, the details of which will be described in section 3.7.

### 3.5.3 Silicon Protective Groups



Ins(1,3,4,6)$P_4$ **(61)**

R = H **(58)** ⟶ R = Bz **(59)**
bis-disiloxane

**Figure 28**

There have been several reports of hydroxyl protection using silicon protective groups. 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (TIPS$Cl_2$) has been used to protect diols. First, [370] the treatment of *myo*-inositol with 2.5 equivalents of TIPS$Cl_2$ in pyridine at room temperature gave the highly crystalline bis-disiloxane **(58)** derivative (Figure 28) in 66% yield. This compound was esterified at the 2- and 5-positions with benzoyl chloride in boiling pyridine, and the silicon protective groups were removed by treatment with aqueous hydrogen fluoride in acetonitrile to give 2,5-di-*O*-benzoyl-*myo*-inositol **(60)** in 96% yield. This tetrol was phosphorylated and deprotected to give Ins(1,3,4,6)$P_4$ **(61)**. [370] Second, when DL-1,2-*O*-cyclohexylidene-*myo*-inositol was treated with $TIPSCl_2$ (1.2 equivalents) in pyridine at room temperature, the 3,4-TIPS derivative was formed in 90% yield. [370] This intermediate could be used for the synthesis of Ins(1,3,4)$P_3$ by further protection and deprotection steps.

The exposed 1,2-diol of the protected DL-3,6-di-*O*-benzyl-4,5-di-*O*-bis (dibenzyloxyphospho)-*myo*-inositol was selectively silylated using triethylsilyl chloride in pyridine at 0°C. The triethylsilyl group was selectively introduced at the equatorial 1-hydroxyl position (95% yield) over the axial 2-hydroxyl position. Benzoylation at the axial 2-position (97% yield) and desilylation with acid provided the 1-hydroxy derivative which was phosphorylated and deprotected to give racemic Ins(1,4,5)$P_3$. [371] *t*-Butyldiphenylsilyl chloride was used to silylate selectively DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol at the 3-hydroxyl group in 60% yield. [372] The 6-position was esterified with (1*S*)-(-)-ω-camphanic acid chloride and the individual diastereoisomers were separated by high performance liquid chromatography, (HPLC). The 1-*O*-silyl derivative was used to synthesise the chiral phospholipid D-*myo*-inositol-1-(sodium 1,2-di-*O*-hexadecanoyl-*sn*-glycer-3-yl-phosphate). The silicon group was removed by treatment with tetrabutylammonium fluoride in tetrahydrofuran (THF).

When D-2,3-*O*-camphor-*myo*-inositol is treated with *t*-butyldiphenylsilyl chloride (1.1 equivalents) in the presence of imidazole in acetonitrile, D-1-*O*-*t*-butyldiphenylsilyl-2,3-*O*-camphor-*myo*-inositol was formed in 30% yield. When 2.2 equivalents of *t*-butyldiphenylsilyl chloride were used, then D-1,4-di-*O*-*t*-butyldiphenylsilyl-2,3-*O*-camphor-*myo*-inositol was formed in 50% yield. [348]

### 3.6 Selective Hydroxyl Protection Using Dibutyltin Oxide

The difference between the reactivities of secondary hydroxyl groups in sugars and cyclitols is small, so inevitably substitution of a polyol leads to several products. There has been a dramatic increase in the use of organotin derivatives, and to a lesser extent organoborane derivatives, to activate selectively, one hydroxyl group over a neighbouring one and provide partially protected *myo*-inositol derivatives which have been difficult to obtain otherwise.

Some of the first reports of selective substitution at one hydroxyl of a vicinal diol, appeared using pyranosides as the source of the 1,2-diol. When the 1,2-diol was suspended in toluene or methanol in the presence of dibutyltin oxide or the bis-alkoxide, water or an alcohol was removed azeotropically, together with the formation of the dibutylstannylene. In non polar solvents such as toluene, dibutylstannylenes exist as dimers (Figure 29). The chemical shifts in the $^{119}Sn$ NMR spectra of carbohydrate stannylenes in $CDCl_3$ are characteristic of a penta-coordinate tin, in which the O-Sn-O angle is approximately 80°, accommodated in a trigonal bipyramid. This topic has been reviewed by S. David and S. Hanessian. [373]

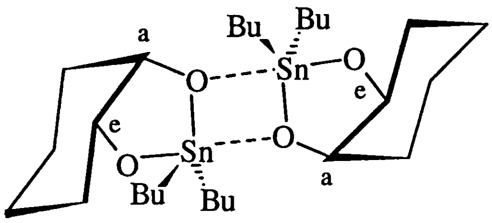



**Figure 29**

**(62)**

Dibutylstannylene in non-polar solvent

The alkylation of 1,2-diols has been mediated by stannylenes in the presence of CsF. When the stannylene has been formed the solvent was evaporated and then dried for several hours *in vacuo* at high temperature (90°C), in the presence of CsF (2.5 equivalents). Anhydrous DMF was added followed by the alkyl halide (for example, benzyl bromide) to give the monoalkylated product in high yield. [374]

Caesium fluoride plays a major role in the selective alkylation reaction. Caesium fluoride has a highly polarisable caesium cation and an electronegative fluoride anion. The high reactivity of the dibutylstannylene acetal activated by Sn-O bonds, is not sufficient to explain the high regioselectivity. However, the polarisable caesium cation may activate the halogen of the alkyl halide. The activation of the Sn-O bond may be caused by the formation of a pentacoordinate complex, because the maximum yield was obtained with 2 equivalents of CsF. Both the caesium cation and the fluoride anion contribute towards the high regioselective and high yielding *O*-monoalkylation of the diol.

There are several examples where CsF has mediated highly regioselective alkylations in the synthesis of *myo*-inositol derivatives. DL-1,2:5,6-Di-*O*-cyclohexylidene-*myo*-inositol was stannylated with dibutyltin oxide across the 3,4-*trans*-diol. This tin complex was activated with caesium fluoride in DMF using benzyl bromide as the alkylating reagent, which provided DL-4-*O*-benzyl-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol **(63)** in 97% yield, (Figure 30). [375] In several examples, the *cis*-1,2-diol of *myo*-inositol derivatives was alkylated in the presence of caesium fluoride. First, DL-

1,4-di-*O*-benzyl-5,6-di-*O*-butanoyl-*myo*-inositol was stannylated using dibutyltin oxide to form the *cis*-stannylene which was alkylated using *p*-methoxybenzyl chloride/potassium iodide together with 2.5 equivalents of CsF in DMF to give DL-1-*O*-*p*-methoxybenzyl-3,6-di-*O*-benzyl-4,5-di-*O*-butanoyl-*myo*-inositol in 85% yield after crystallisation. [358] Second, allylation of DL-1,4-di-*O*-allyl-6-*O*-benzyl-5-*O*-*p*-methoxybenzyl-*myo*-inositol using allyl bromide under similar conditions provided DL-1,3,4-tri-*O*-allyl-6-*O*-benzyl-5-*O*-*p*-methoxybenzyl-*myo*-inositol derivative in 83% yield. [359] Third, allylation of D-3,4,5-tri-*O*-allyl-6-*O*-benzyl-*myo*-inositol *via* the *cis*-1,2-*O*-stannylene acetal with allyl bromide together with CsF provided the D-1,3,4,5-tetra-*O*-allyl-6-*O*-benzyl-*myo*-inositol derivative in 95% yield. [376] These examples provide high yielding and highly regioselective mono-alkylation of a diol, which otherwise would be difficult to obtain under the usual conditions described for the individual protective groups.

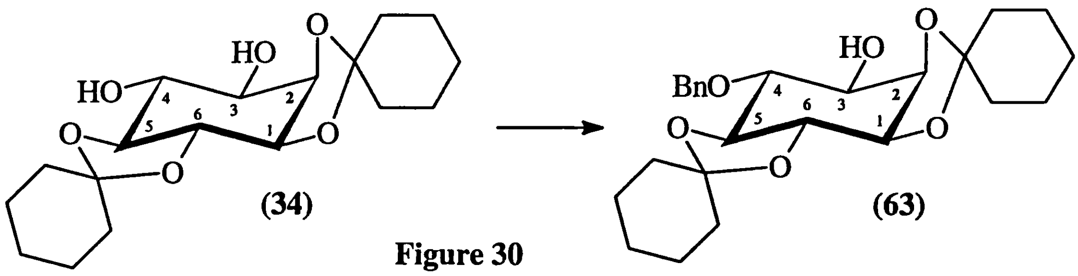



**Figure 30**

The last few years have seen many examples for the selective alkylation of polyhydroxy compounds, especially *myo*-inositol, and its derivatives. From previous work, it has been demonstrated that alkylation of the *cis*-1,2-*O*-stannylene derivative in a six membered ring provides preferential alkylation at the equatorial site. [358] The solvent of choice in most of the examples was toluene, in the presence of tetrabutylammonium bromide or iodide. Roy Gigg and his coworkers [377] used acetonitrile as the solvent which was refluxed in a solid/liquid soup of *myo*-inositol, dibutyltin oxide and tetrabutylammonium bromide in the presence of molecular sieves in a soxhlet thimble to remove the water which was formed. The reaction of *myo*-inositol with dibutyltin oxide, tetrabutylammonium bromide (2 equivalents each) and a large excess of allyl bromide in refluxing acetonitrile, gave many products which could be separated by chromatography, but no major product was formed. The reaction of *myo*-inositol with dibutyltin oxide, tetrabutylammonium bromide (3 equivalents each) and a large excess of allyl bromide in refluxing acetonitrile gave fewer products. Several ether soluble derivatives were formed including DL-1,3,4,5-tetra-*O*-allyl-*myo*-inositol, 1,3,4,6-tetra-*O*-allyl-*myo*-inositol, and DL-1,2,3,4-tetra-*O*-allyl-*myo*-inositol in small quantities. However, the major product formed was the water soluble DL-1,3,4-tri-*O*-allyl-*myo*-inositol which was isolated in 44% yield, together with a small portion of 1,3,5-tri-*O*-allyl-*myo*-inositol and several mono- and di-*O*-allyl compounds which were characterised as their known di-*O*-isopropylidene derivatives. This major product has tremendous potential for the synthesis of several *myo*-inositol phosphate analogues in a one pot high yielding reaction for the selective protection of 3 out of the 6 hydroxyl groups of *myo*-inositol. When 5 equivalents of dibutyl tin oxide were used under similar conditions, fewer allylated products were formed and only a small amount of penta-*O*-allyl-*myo*-inositol was formed. There were two major products: 1,3,4,6-tetra-*O*-allyl-*myo*-inositol, (25%) and DL-1,3,4,5-tetra-*O*-allyl-*myo*-inositol, (26%), together with other minor products. The tetra-*O*-allyl derivatives have been used to prepare their respective tetrakisphosphates in only a few transformations starting from *myo*-inositol. [377]

The tetrabutylammonium bromide plays a critical role in the reaction, being a very effective catalyst. Without the tetrabutylammonium bromide the reaction would not proceed or go to completion. It has been suggested [378] that the co-ordination of the halide anion to the tin of the stannylene complex enhances the nucleophilicity of one of the bound oxygen atoms in some unknown manner. The polar aprotic nature of acetonitrile may also influence the nucleophilicity of the oxygen at one of the tin atoms.

In a more recent paper, Gigg and his coworkers [379] have selectively alkylated racemic 1,2-*O*-isopropylidene-*myo*-inositol with excess benzyl bromide, dibutyltin oxide and tetrabutylammonium bromide in refluxing acetonitrile to give DL-3,4,6-tri-*O*-benzyl-1,2-*O*-isopropylidene-*myo*-inositol in 40% yield and DL-3,5,6-tri-*O*-benzyl-1,2-*O*-isopropylidene-*myo*-inositol in 30% yield together with four minor components. When 2 equivalents of dibutyltin oxide were used, DL-3,6-di-*O*-benzyl-1,2-*O*-isopropylidene-*myo*-inositol was formed in *ca.* 45% yield which is less cumbersome than the 5-step transformation first devised by Gigg and his coworkers. [349]

Selective *p*-methoxybenzylation of DL-1,6-di-*O*-benzyl-2,3-*O*-isopropylidene-*myo*-inositol with dibutyltin oxide and excess *p*-methoxybenzyl chloride in the presence of tetrabutylammonium bromide gave a mixture of the 4-*O*-*p*-methoxybenzyl derivative in 64% yield and the 5-*O*-*p*-methoxybenzyl derivative in 23% yield, indicating a selectivity of 3:1 for the 4-position over the 5-position. Under the same conditions, but replacing *p*-methoxybenzyl chloride with allyl bromide no regiospecificity was observed. [380] The use of the dibutylstannylene acetal to regioselectively alkylate *myo*-inositol derivatives has greatly reduced the number of transformations needed to provide a suitable phosphorylation precursor.

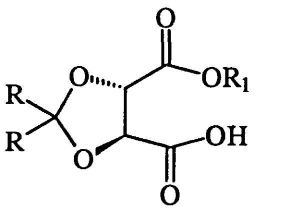
### 3.7 Resolution of *myo*-Inositol Derivatives

There have been several methods used to resolve *myo*-inositol phosphate precursors. The majority of these methods rely on the derivatisation of the racemate with a chiral acid derivative to form a pair of diastereoisomeric esters. These esters may be separated by silica gel chromatography or by crystallisation. HPLC analysis has been used to determine the diastereomeric purity and basic hydrolysis of the individual diastereoisomers regenerates the corresponding enantiomers.

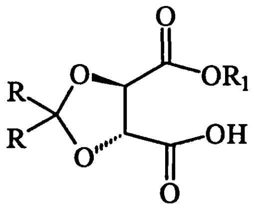
Two other methods have been used, albeit infrequently, for the resolution of *myo*-inositol derivatives. First, using chiral derivatives of carbohydrates and second, employing a chiral HPLC column. Russian scientists have used D-mannose orthoacetate [381] and D-glucose orthoacetate [381] derivatives to resolve racemates. The diastereoisomeric intermediates were separated by silica gel chromatography or selective crystallisation. In the early years of *myo*-inositol phosphate synthesis, a chiral HPLC column was used to separate DL-5,6-di-*O*-benzyl-3,4-di-*O*-*p*-methoxybenzyl-*myo*-inositol possessing a free 1,2-dihydroxy moiety. Highly efficient resolution of this racemate was accomplished by using a stainless steel tube packed with the chiral cellulose 3,5-dimethylphenylcarbamate derivative, supported on silica gel. Eluting with propanol/hexane (1:5) gave the L-5,6-di-*O*-benzyl-3,4-di-*O*-*p*-methoxybenzyl-*myo*-inositol (retention time 15min) and the D-isomer (retention time 25min). The latter enantiomer was used to synthesise D-Ins(1,3,4)$P_3$. [350]

The main chiral acids or acid chlorides that have been used for the resolution of *myo*-inositol derivatives are: (-)-menthoxyacetyl chloride **(66)**, D- and L-tartaric acid monoester **(64,65)** and finally (1*S*)-(-)-ω-camphanic acid chloride **(67)** and (1*R*)-(+)-ω -camphanic acid chloride **(68)**. One example of a carbamate derivative using *R*-(+)-(1-phenylethyl)isocyanate **(69)** has also been employed (Figure 31). The most popular method, by far, for resolving racemates has been the derivatisation by means of *S*-(-)-camphanate esters. The use of enzymes (such as lipases and esterases) for the synthesis of *myo*-inositol phosphate precursors has also been utilised and will be discussed later.

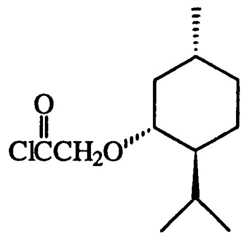
It has been discussed above that benzoylation of *myo*-inositol provides a series of protected intermediates, including 1,3,4,5-tetra-*O*-benzoyl-*myo*-inositol and the *meso* derivative 1,3,5-tri-*O*-benzoyl-*myo*-inositol.[369] The *meso* compound was selectively acylated with a chiral acid derivative to provide protection at the 1,3,4,5-positions. The readily available chiral acids such as (-)-menthoxyacetyl chloride, (-)-menthoxycarbonyl chloride, amino acid mixed anhydrides and isocyanates were unable to obtain useful selectivities. However, kinetic resolution of 1,3,5-tri-*O*-benzoyl-*myo*-inositol (1 equivalent) using methyl hydrogen 2,3-*O*-cyclohexylidene-D-tartrate in the presence of methanesulphonyl chloride (1.1 equivalents), *N*-methylmorpholine (2.5 equivalents) and a catalytic amount of DMAP in THF at 0°C provided the diastereoisomeric monotartrates in an optimised yield of 62% with a diastereoisomeric excess of 96% and 1,3,5-tri-*O*-benzoyl-*myo*-inositol was recovered in 28% yield. The methyl hydrogen 2,3-*O*-cyclohexylidene-L-tartrate derivative provided the L-1,3,4,5-tetra-*O*-acylated *myo*-inositol derivative with the same diastereoisomeric excess.



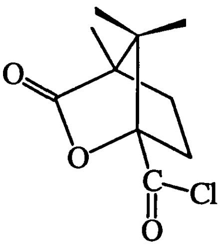

R-R = $(CH_2)_5$, $R_1 = CH_3$
Methyl hydrogen 2,3-*O*-cyclohexylidene-D-tartrate **(64)**



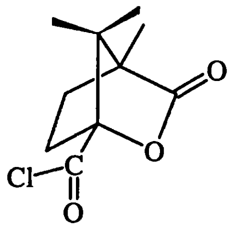

R-R = $(CH_2)_5$, $R_1 = CH_3$
Methyl hydrogen 2,3-*O*-cyclohexylidene-L-tartrate **(65)**



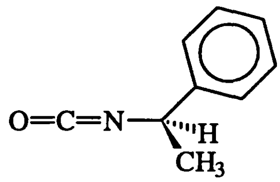

*l*-Menthoxyacetyl chloride **(66)**




(1*S*)-(-)-Camphanic acid chloride **(67)**




(1*R*)-(+)-Camphanic acid chloride **(68)**




*R*-(+)-1-Phenylethyl isocyanate **(69)**

**Figure 31**

The major use of (-)-menthoxyacetyl chloride will be discussed in the first synthesis of D-Ins(1,4,5)$P_3$. In a later synthesis of D-Ins(1,4,5)$P_3$, racemic 1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol was derivatised as the 3,4-di-*O*-menthoxyacetate, to provide two diastereoisomers with quite different chromatographic mobilities on TLC [ether/hexane (1:3) $R_f$ = 0.87 for D-(-)-1,2:5,6-di-*O*-cyclohexylidene-3,4-di-*O*-menthoxyacetyl-*myo*-inositol and for the slower moving diastereoisomer, L-1,2:5,6-di-

*O*-cyclohexylidene-3,4-di-*O*-menthoxyacetyl-*myo*-inositol an $R_f$ = 0.74. [382] The L-derivative was used to prepare D-Ins(1,4,5)$P_3$.

Vasella and coworkers [352] have synthesised both enantiomers of Ins(1,3,4,5)$P_4$, *via* DL-4-*O*-benzyl-2-*O*-*t*-butyldimethylsilyl-*myo*-inositol orthoformate. This compound was treated with (*R*)-(+)-1-(phenylethyl)isocyanate in the presence of butyl lithium at -78°C to provide a mixture of diastereoisomers and unreacted starting material. In order to separate the diastereoisomers the product was desilylated and the diastereoisomers were separated by medium pressure chromatography and the racemate was recycled. Further derivatisation of the individual diastereoisomers gave D- and L-2,6-di-*O*-benzyl-*myo*-inositol which were used to synthesise D- and L-Ins(1,3,4,5)$P_4$.

The enantiomers of camphanic acid are the most popular resolving agents to date for the synthesis of chiral *myo*-inositol phosphate precursors. Until recently, only 1*S*-(-)-(ω)-camphanic acid chloride **(67)** was available, but in 1989 the 1*R*-(+)-(ω)-camphanic acid chloride **(68)** became commercially available from Fluka. However, the 1*S*-(-)-(ω)-camphanic acid chloride is the most widely used because 1*R*-(+)-(ω)-camphanic acid chloride is very expensive, (250mg, £25.70, Fluka 1993/94). 1*S*-(-)-(ω)-Camphanic acid chloride is a highly crystalline reagent which is available in >98% optical purity. The camphanate esters that are formed may be separated by column chromatography (when the $R_f$ values for the two diastereoisomers are different) or more appropriately by crystallisation of one diastereoisomer from the mixture. At least one of the camphanate derivatives is usually highly crystalline and the absolute configuration of the diastereoisomer may be evaluated by X-ray crystallography. The chiral camphanic acid may be recovered from the aqueous layer after saponification, by acidification followed by extraction with dichloromethane.

The first significant use of 1*S*-(-)-(ω)-camphanic acid chloride [383] was in the resolution of DL-1,2,4-tri-*O*-benzyl-5,6-*O*-isopropylidene-*myo*-inositol (Figure 32) to give a mixture of 3-*O*-camphanate ester derivatives. After work-up and evaporation, the remaining syrup was dissolved in ether and crystals appeared rapidly. After separating the crystals, the remaining syrup was diluted with methanol and more crystals appeared, which contained one diastereoisomer only in 86% yield. This highly crystalline compound was subsequently found to be D-1,2,4-tri-*O*-benzyl-3-*O*-(-)-camphanoyl-5,6-*O*-isopropylidene-*myo*-inositol **(72)** which was the wrong diastereoisomer for the synthesis of D-Ins(1,4,5)$P_3$. Later, with the availability of 1*R*-(+)-(ω)-camphanic acid chloride, the mother liquor containing mainly the non-crystalline L-1,2,4-tri-*O*-benzyl-3-*O*-(-)-camphanoyl-5,6-*O*-isopropylidene-*myo*-inositol **(71)** was saponified to give L-1,2,4-tri-*O*-benzyl-5,6-*O*-isopropylidene-*myo*-inositol. This compound was treated with 1*R*-(+)-(ω)-camphanic acid chloride to give the crystalline L-1,2,4-tri-*O*-benzyl-3-*O*-(+)-camphanoyl-5,6-*O*-isopropylidene-*myo*-inositol **(73)**. The pure diastereoisomer was crystallised from ether in 80% yield. Saponification of this L-derivative followed by deacetonation provided D-2,3,6-tri-*O*-benzyl-*myo*-inositol which was used to synthesise D-Ins(1,4,5)$P_3$. [384]

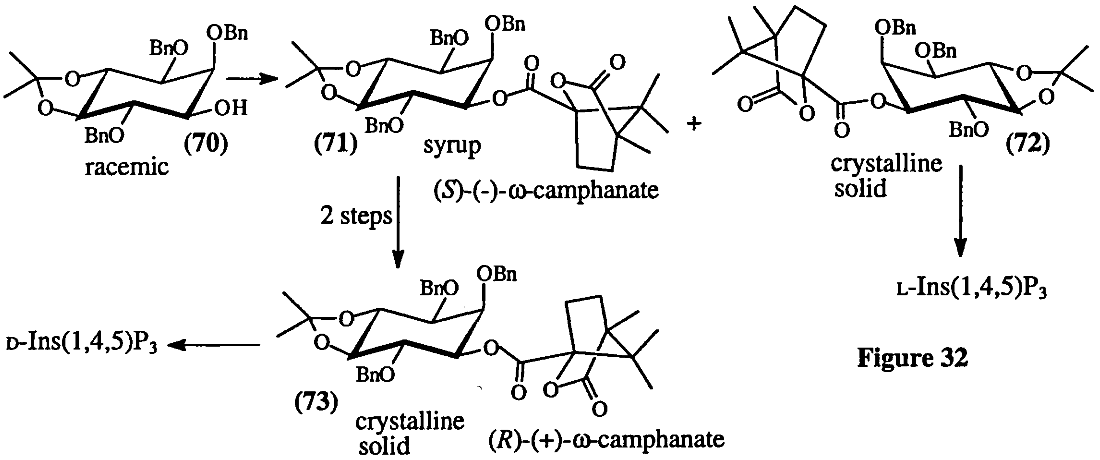



**Figure 32**

In the synthesis of both enantiomers of 2-deoxy-2,2-difluoro-*myo*-inositol-1,4,5-trisphosphate, 1*S*-(-)-(ω)-camphanic acid chloride was employed to resolve the intermediate DL-3,6-di-*O*-benzyl-2-deoxy-2,2-difluoro-4,5-*O*-isopropylidene-*myo*-inositol **(74)** (Figure 33). [385] Thus, treatment of the racemic derivative **(74)** with 1*S*-(-)-(ω)-camphanic acid chloride provided two diastereoisomers which were separated by silica gel chromatography using dichloromethane as the eluting solvent.

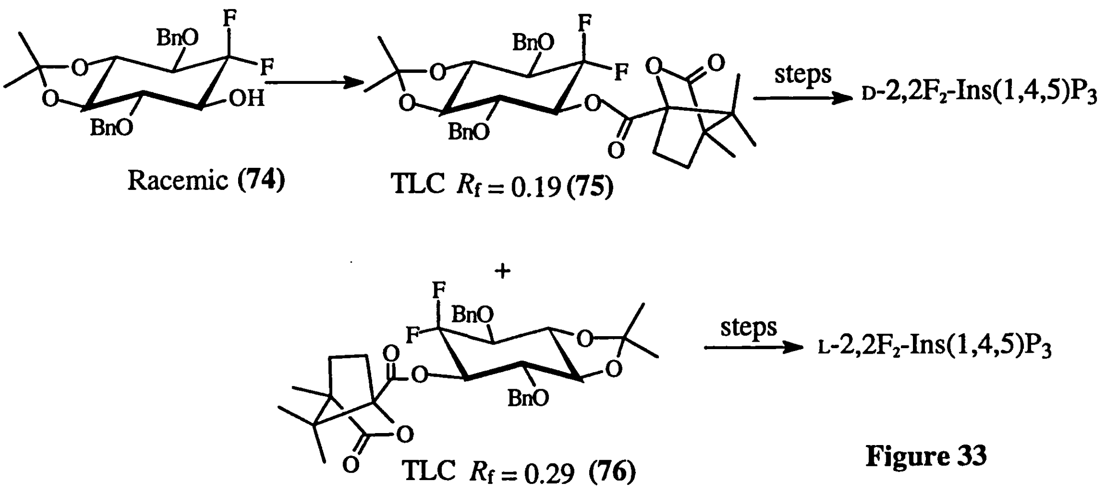



**Figure 33**

The slower moving diastereoisomer (**75**) on TLC, $R_f = 0.19$ (60% yield) provided the derivative which was suitable for single X-ray crystallography [385] and was subsequently found to be the intermediate for the synthesis of D-2-deoxy-2,2-difluoro-*myo*-inositol-1,4,5-trisphosphate. The faster moving diastereoisomer, $R_f = 0.29$ (64% yield) (**76**) provided the intermediate for the synthesis of L-2-deoxy-2,2-difluoro-*myo*-inositol-1,4,5-trisphosphate.

In another example, DL-1-*O*-allyl-2,3,6-tri-*O*-benzyl-*myo*-inositol [358] was treated with 1S-(-)-(ω)-camphanic acid chloride to give the 4,5-di-*O*-camphanoyl derivative. After work-up the syrup was taken up in ether and kept at -20°C overnight, after which time crystals had appeared. These were filtered and the mother liquor was redissolved in ether-methanol to give more crystals of D-(+)-1-*O*-allyl-2,3,6-tri-*O*-benzyl-4,5-di-*O*-camphanoyl-*myo*-inositol in 74% yield. Surprisingly, after leaving the mother liquor for several days at -20°C a solid was formed. The solid was redissolved in warm ether and after 2 days the other diastereoisomer crystallised in 80% yield. The D-derivative was used to synthesise D-*myo*-inositol-4,5-bisphosphate 1-phosphorothioate.

The derivative, DL-1,3,4-tri-*O*-allyl-2-*O*-benzyl-*myo*-inositol was treated with 1*S*-(-)-(ω)-camphanic acid chloride to give a mixture of bis-camphanates. The two diastereoisomers had only slightly different mobilities by TLC, differing by an $R_f$ = 0.05. Crystallisation of the L-5,6-di-*O*-camphanoyl derivative (84% yield, $R_f = 0.4$) provided a suitable intermediate for the synthesis of L-Ins(1,3,4)$P_3$. The other diastereoisomer ($R_f = 0.45$) was obtained by column chromatography of the mother liquors and provided a suitable intermediate for the synthesis of D-Ins(1,3,4)$P_3$. [386] Finally, a one pot allylation of *myo*-inositol, (described in section 3.6) provided 1,3,4,5-tetra-*O*-allyl-*myo*-inositol in 26% yield. Treatment of this diol with 1*S*-(-)-(ω)-camphanic acid chloride gave a mixture of 2,6-di-*O*-camphanates with different mobilities by TLC ($R_f$ = 0.65 and 0.70, ether-light petroleum, 4:1). The slower moving bis-camphanate, $R_f = 0.65$ was crystallised directly from the mixture of diaster eoisomers with ether-petroleum ether (1:1), to give the L-2,6-di-*O*-camphanoyl-1,3,4,5-tetra-*O*-allyl-*myo*-inositol. The other diastereoisomer ($R_f = 0.70$) was obtained by column chromatography of the mother liquors. These chiral intermediates were used to synthesise both enantiomers of Ins(1,3,4,5)$P_4$. [387] These examples represent successful applications of 1*S*-(-)-(ω)-camphanic acid chloride and its enantiomer in the synthesis of *myo*-inositol phosphate derivatives.

### 3.7.1 The Use of Enzymes to Resolve *myo*-Inositol Phosphate Derivatives

The first example in which enzymes were used to resolve *myo*-inositol phosphate precursors, appeared in 1988 by the Vasella group. [352] The *meso*-derivative 4,6-di-*O*-butanoyl-*myo*-inositol-orthoformate, **(77)** (prepared in 3 steps from the orthoformate) (Figure 34) was hydrolysed enantiospecifically by pig liver esterase (PLE) to give L-4-*O*-butanoyl-*myo*-inositol-orthoformate **(78)** in 83% yield and 95% enantiomeric excess. One recrystallisation followed by a further 3 steps converted this intermediate into optically pure L-2,6-di-*O*-benzyl-*myo*-inositol. However, 6 more protection-deprotection steps were required to convert L-4-*O*-butanoyl-*myo*-inositol-orthoformate into D-2,6-di-*O*-benzyl-*myo*-inositol. Both compounds were phosphorylated and deprotected to give L- and D-Ins(1,3,4,5)$P_4$ respectively.

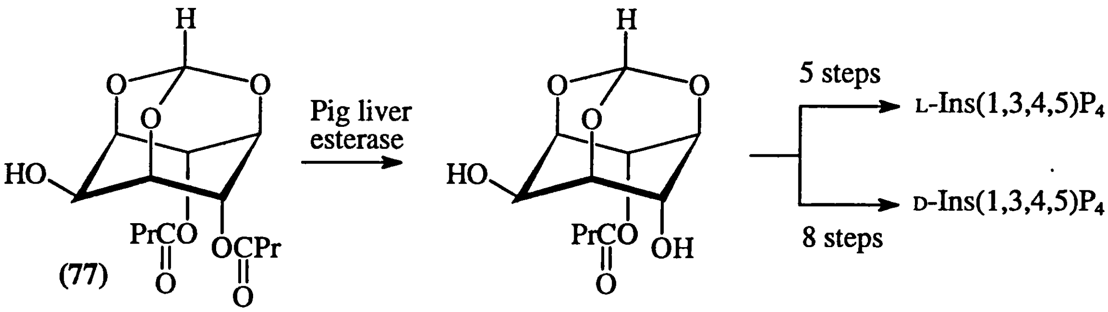
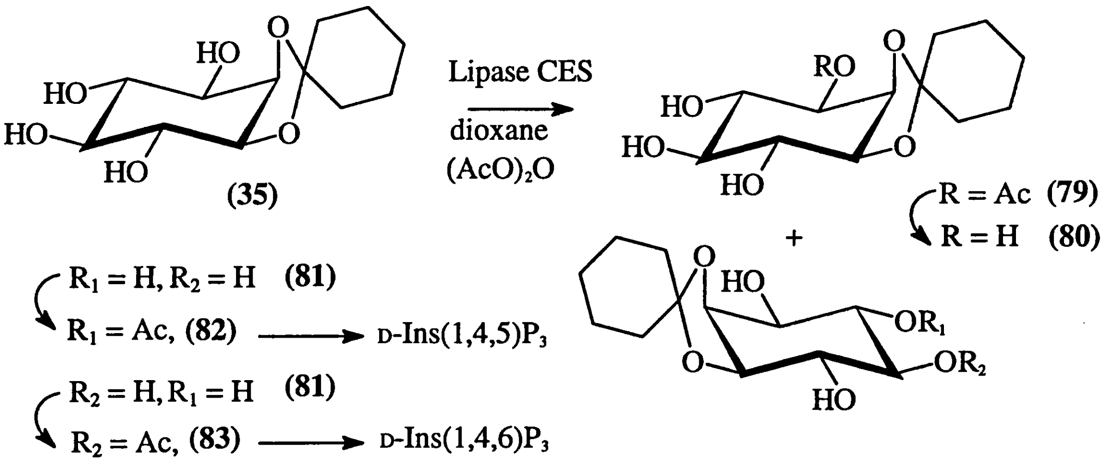



**Figure 34** L-4-*O*-Butyryl-*myo*-inositol orthoformate **(78)**

Pr = Propyl

The major contribution to the synthesis of chiral *myo*-inositol phosphate precursors using enzymes has come from the laboratory of Professor C.-S. Chen. [376] The treatment of *myo*-inositol with 1-ethoxycyclohexene provided three racemic di-*O*-cyclohexylidene-*myo*-inositol derivatives. Two of these, DL-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol and DL-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol were acylated to form their respective di-*O*-acetates and di-*O*-butyrates. By exposure of the individual compounds to a number of esterases and many lipases and proteases it was found that DL-3,6-di-*O*-acyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositols were resistant to deacylation whereas the DL-3,4-di-*O*-acyl derivatives were deacylated by most of the enzymes tested. Finally, two enzymes, cholesterol esterase and porcine pancreatic lipase showed high degrees of stereochemical discrimination. The length of the acyl group was also critical in the optical purity of the product, with the 3,4-di-*O*-butanoyl derivative providing a higher enantiomeric excess than the 3,4-di-*O*-acetate. The problem of a slow reaction rate with di-*O*-acyl derivatives was overcome by using DL-4-*O*-butanoyl-1,2:5,6-di-*O*-cyclohexylidene *myo*-inositol, a compound with minimised steric congestion and improved solubility. The exposure of DL-4-*O*-butanoyl-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol (15g) in phosphate buffer (0.1M), containing crude porcine pancreatic lipase for 2days at room temperature provided two compounds. First, D-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol (5.6g, 88% enantiomeric excess) and L-4-*O*-butanoyl-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol (6.5g, 95% enantiomeric excess). Both derivatives were optically pure after one recrystallisation. The latter derivative, was deacylated and selectively benzylated to give D-(-)-6-*O*-benzyl-2,3:4,5-di-*O*-cyclohexylidene-*myo*-inositol. The less stable *trans*-acetal was removed under acid conditions to give D-6-*O*-benzyl-2,3-*O*-cyclohexylidene-*myo*-inositol, which was phosphorylated and deprotected to afford D-Ins(1,4,5)$P_3$. Further manipulation of provided intermediates for the synthesis of D-Ins(1,3,4)$P_3$, and D-Ins(1,3,4,5)$P_4$. [376]




Ac = acetate

For **(80)** the optical rotation is known

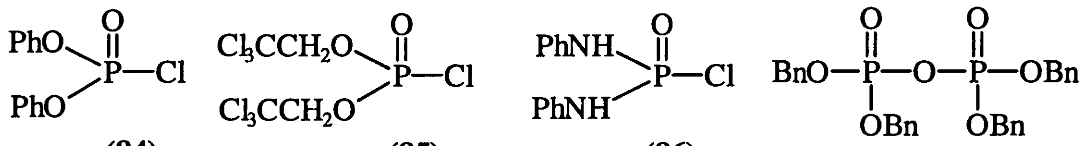
**Figure 35**

The kinetic resolution of DL-1,2-*O*-cyclohexylidene-*myo*-inositol has been carried out by enzyme-catalysed enantioselective esterification using two hydrolytic enzymes, from *Pseudomonas spp.* (Amano Lipase P and Lipase CES). [388] DL-1,2-*O*-Cyclohexylidene-*myo*-inositol **(35)** was dissolved in dry dioxane, together with acetic anhydride and the enzyme. Lipase P and Lipase CES acylated selectively the hydroxyl group at C-3 of the D-enantiomer (Figure 35). Lipase CES provided the highest optically pure derivatives, in 98% chemical yield and 98% enantiomeric excess for D-3-*O*-acetyl-1,2-*O*-cyclohexylidene-*myo*-inositol **(79)** and 98% chemical yield and >99% enantiomeric excess for L-1,2-*O*-cyclohexylidene-*myo*-inositol **(81)**. L-1,2-*O*-Cyclohexylidene-*myo*-inositol was acylated selectively with acetic anhydride (6 equivalents) in the presence of dimethylacetamide (DMA) at room temperature with 4Å sieves to afford 4-*O*-acylated **(82)** and 5-*O*-acylated **(83)** products in a ratio of

1:1, in 74% yield. The mixture of acetylated products was subject to phosphorylation and separated by column chromatography. The phosphorylated D-6-*O*-acetyl-2,3-*O*-cyclohexylidene-*myo*-inositol derivative provided D-Ins(1,4,5)$P_3$ after deprotection, in a small number of steps, whilst the phosphorylated D-5-*O*-acetyl-2,3-*O*-cyclohexylidene-*myo*-inositol derivative gave D-Ins(1,4,6)$P_3$ in a few steps. Phosphorylation was carried out with Ozaki's reagent *via* the P(III) method outlined in the next section.

### 3.8 Phosphorylation Methods

In the early days of inositol phosphate synthesis, it was reported that phosphorylation of the hydroxyl groups was quite difficult. There are three main reasons for this. First, the reactivity of the hydroxyl functions of *myo*-inositols is generally low compared with other alcohols and nucleotides because of steric crowding and hydrogen bonding; thus efficient phosphorylation of a vicinal 4,5-diol of Ins(1,4,5)$P_3$ or a vicinal 3,4,5-triol of Ins(1,3,4,5)$P_4$ must be achieved for a successful synthesis. This can only be achieved using a suitable P(III) reagent or a highly reactive P(V) reagent. Second, strongly acidic and basic conditions must be avoided to reduce the chance of phosphate migration to a neighbouring hydroxyl group. Third, the resulting phosphate triester may form cyclic phosphates if the hydroxyl groups are not suitably protected.



(84) (85) (86) (87)

**Figure 36**

Four P(V) reagents have been used in the synthesis of *myo*-inositol phosphates, (Figure 36). These include, diphenyl phosphochloridate **(84)**, bis(2,2,2-trichloroethyl) phosphochloridate **(85)**, dianilidophosphoryl chloride **(86)** and tetrabenzyl pyrophosphate **(87)**. Diphenyl phosphochloridate has been used to synthesise racemic *myo*-inositol 1-phosphate, Ins(1)P. [389] The phosphorylation precursor, 1,2,4,5,6-penta-*O*-benzyl-*myo*-inositol, failed to react with either dibenzyl chlorophosphate or tetrabenzyl pyrophosphate, under the conditions used. The hydroxyl group was finally phosphorylated with diphenyl chlorophosphate in triethylamine and DMAP, followed by transesterification with the anion of benzyl alcohol using sodium hydride as base in THF to give the required hepta-*O*-benzyl-*myo*-inositol-1-phosphate derivative in 83%

yield. Hydrogenolysis of all seven benzyl protective groups gave racemic *myo*-inositol-1-phosphate, which was isolated as its crystalline biscyclohexylammonium salt in 95% yield. Dianilidophosphoryl chloride has been used in the first synthesis of Ins(1,4,5)$P_3$. However, deprotection methods were not entirely successful so its use by Ozaki and coworkers was discontinued. The synthesis of Ins(1,4,5)$P_3$ will be discussed in section (3.9).

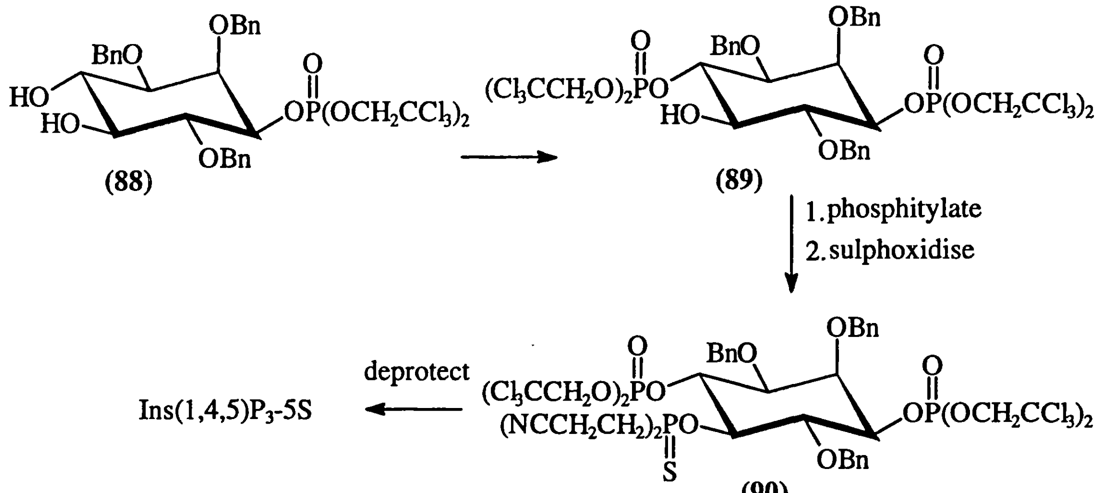
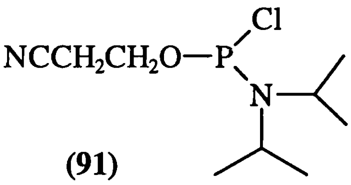
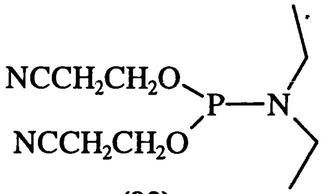


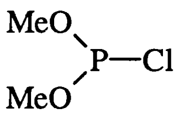

**Figure 37**

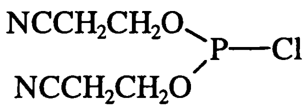
A combination of P(III) and P(V) chemistry provided a synthesis of racemic *myo*-inositol 1,4-bisphosphate-5-phosphorothioate, [390] a close analogue of Ins(1,4,5)$P_3$ which is resistant to the action of the 5-phosphatase enzyme. The racemic diol DL-1,2,4-tri-*O*-benzyl-*myo*-inositol-3-[di(2,2,2-trichloroethyl) phosphate] **(88)** (Figure 37) was monophosphorylated with the sterically hindered reagent, bis(2,2,2-trichloroethyl) phosphochloridate in 29% yield, leaving the 5-hydroxyl group exposed **(89)**. Phosphitylation of the 5-hydroxyl group was accomplished with *N,N*-diisopropylamino(2-cyanoethoxy)chlorophosphine (see below) to give the phosphoramidite. The reaction of the phosphoramidite *in situ* with 2-cyanoethanol and tetrazole gave the phosphite which was sulphoxidised using sulphur in pyridine to provide the fully protected 1,4-bisphosphate-5-phosphorothioate derivative **(90)**. A one step deprotection using sodium in liquid ammonia removed all the protective groups to give the racemic 5-phosphorothioate derivative in 88% yield, after purification by ion exchange chromatography.

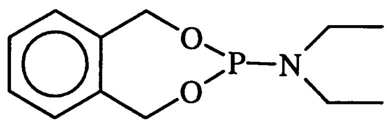
The P(V) reagent tetrabenzyl pyrophosphate has been used to synthesise Ins(1,4,5)$P_3$, Ins(1,3,4)$P_3$ and several bisphosphates. [364] The reagent is easy to handle and a fairly stable crystalline compound of relatively low toxicity. The phosphorylation of DL-2,4,5-tri-*O*-benzyl-*myo*-inositol was carried out in THF at 60°C with sodium hydride as base, to form the alkoxide, which was then reacted with tetrabenzyl pyrophosphate to form the fully protected Ins(1,3,4)$P_3$ derivative. The benzyl protective groups were removed by hydrogenolysis. DL-2,4-Di-*O*-benzyl-*myo*-inositol was phosphorylated under the same conditions as for DL-2,4,5-tri-*O*-benzyl-*myo*-inositol, in 66% yield and hydrogenolysis afforded Ins(1,3,4,5)$P_4$, in 88% yield. [353]


**(91)**


**(92)**


**(93)**


**(94)**


**(95)**

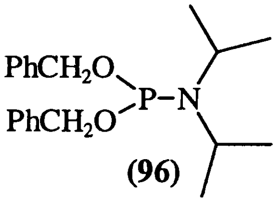

**(96)**

**Figure 38**

The second method of introducing a phosphate moiety into the *myo*-inositol ring involves the reaction of the hydroxyl group with a P(III) reagent (Figure 38), which is more reactive than its P(V) counterpart. Oxidation of the P(III) intermediate with *t*-butylhydroperoxide [358] or *m*-CPBA [375] gives a phosphate triester intermediate whereas sulphoxidation with sulphur in pyridine [391] or phenacetyl disulphide [392] provides the phosphorothioate triester. The first P(III) reagent used to phosphitylate *myo*-inositol derivatives was *N,N*-diisopropylamino(2-cyanoethoxy)chlorophosphine **(91)** which will be fully described in Potter's synthesis of racemic Ins(1,4,5)$P_3$. Reese and Ward [346] introduced another reagent, bis(2-cyanoethoxy)diethylaminophosphine **(92)** which was used to phosphitylate the intermediate, DL-1,2-*O*-cyclopentylidene-4-*O*-(2,7-dibromo-9-phenylxanthen-9-yl)-*myo*-inositol in the presence of 1*H*-tetrazole and dichloromethane as the solvent. The 1,4,5-trisphosphite triester was oxidised with *t*-butylhydroperoxide to give the 1,4,5-trisphosphate and the product was purified by column chromatography. Total deprotection of this intermediate was accomplished in a two step process. First, the base labile cyanoethoxy groups were removed with $N^1$,$N^1$,$N^3$,$N^3$-tetramethylguanidine in ethanol-water (4:1 v/v) at 37°C for 24h, then at room temperature for 48h to give the $N^1$,$N^1$,$N^3$,$N^3$-tetramethylguanidium salt. The cyclopentylidene acetal and 4-*O*-(2,7-dibromo-9-phenylxanthen-9-yl) groups were removed in *ca.* 1h when the product was dissolved in water (pH ~ 6) at room temperature.

Meek and coworkers [393] used dimethoxychlorophosphine **(93)** in the presence of *N,N*-diisopropylethylamine and dichloromethane as solvent, to phosphitylate the four vicinal hydroxyl groups of DL-1,2-*O*-isopropylidene-*myo*-inositol. Hydrogen peroxide was used to oxidise the P(III) to the P(V) phosphate intermediate. The eight methyl groups of DL-1,2-*O*-isopropylidene-3,4,5,6-tetrakis(dimethoxyphospho)-*myo*-inositol were removed using excess bromotrimethylsilane in dry dichloromethane, followed by stirring in water with the autocatalysed hydrolysis of the isopropylidene group, to provide DL-Ins(1,4,5,6)$P_4$. Reagent **(93)** was also used to phosphitylate DL-2,4-di-*O*-benzoyl-*myo*-inositol which was oxidised in the same manner to give the 1,3,4,5-phosphorylated derivative and the methyl groups were removed using 30% hydrogen bromide in acetic acid followed by basic hydrolysis of the benzoate esters to give DL-Ins(1,3,4,5)$P_4$.

Van Boom and coworkers [394] have used several phosphitylating reagents, one being the monofunctional bis(2-cyanoethoxy)chlorophosphine **(94)** which was used to prepare racemic Ins(1,3,4)$P_3$ using *N,N*-diisopropylethylamine as base and acetonitrile as solvent, to phosphitylate DL-2,4,5-tri-*O*-benzyl-*myo*-inositol. The phosphite triester was oxidised with *t*-butylhydroperoxide to give the protected phosphate triester. The base-labile cyanoethoxy groups were deprotected by ammonolysis followed by sodium hydroxide (0.2N) in dioxane/methanol/water at 50°C and neutralised with Dowex 50W $H^+$ form. The benzyl groups were cleaved by catalytic reduction using palladium on carbon under hydrogen to give racemic Ins(1,3,4)$P_3$ as a free acid in 80% yield.

Ozaki and coworkers have also used several phosphitylating reagents. However, over the past few years they have employed a very effective phosphitylating reagent, *o*-xylene-*N,N*-diethylaminophosphine **(95)**. This was easily obtained in a one pot reaction with *o*-xylenediol and hexaethylphosphorustriamide to give the product. Thus, treatment of L-1,2,4-tri-*O*-benzyl-*myo*-inositol with *o*-xylene-*N,N*-diethylamino phosphine in the presence of 1*H*-tetrazole followed by the addition of *m*CPBA afforded the protected tris-phosphate in 97% yield. Hydrogenolysis in methanol and 5% palladium on carbon afforded D-Ins(1,4,5)$P_3$ in quantitative yield as its potassium salt.

Finally, the most popular reagent to date is bis(benzyloxy)diisopropylaminophosphine **(96)** which was first used by Fraser-Reid and coworkers [375] and subsequently by many other groups. Essentially this reagent is employed in the same way as Ozaki's reagent, and Fraser-Reid used it to synthesise racemic Ins(1,4,5)$P_3$. Vasella and coworkers [352] also used the reagent to phosphitylate D-and L-2,4-di-*O*-benzyl-*myo*-inositol in the presence of 1*H*-tetrazole and acetonitrile as solvent. The intermediate tetrakisphosphite was oxidised with *m*CPBA and deprotected by hydrogenolysis with palladium on carbon to give L- and D-Ins(1,3,4,5)$P_4$ respectively in 76% yield as the cyclohexylammonium salt.

At present the 1*H*-tetrazole catalysed phosphitylation of hydroxyl groups is the most popular and most efficient method for introducing phosphate and phosphorothioate groups into the *myo*-inositol ring. Further improvements will not be necessary because yields are approaching quantitative.

### 3.9 Six Approaches to the Synthesis of Ins(1,4,5)$P_3$

#### 3.9.1 Ozaki's Synthesis of D-Ins(1,4,5)$P_3$ from *myo*-Inositol

Ozaki's synthesis started with DL-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol **(32)** which was prepared in one step by treatment of *myo*-inositol with 1-ethoxycyclohexene in DMF with toluene-*p*-sulphonic acid as a catalyst. This intermediate was crystallised from the mixture which also contained DL-1,2:3,4- and DL-1,2:5,6-di-*O*-cyclohexylidene acetals. Treatment of the mother liquor with ethanolic toluene-*p*-sulphonic acid resulted in precipitation of DL-1,2-*O*-cyclohexylidene-*myo*-inositol which was then subjected to cyclohexyledination as described for *myo*-inositol in order to produce more DL-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol. The two hydroxyl groups were benzylated using benzyl chloride in DMF and sodium hydride as base, to afford DL-3,6-di-*O*-benzyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol **(97)** in 90% yield. The less stable 4,5-*trans*-acetal was removed using ethylene glycol in chloroform in the presence of toluene-*p*-sulphonic acid as an acid catalyst to give the exposed 4,5-diol **(98)** in 80% yield. Allylation of the diol using allyl bromide and sodium hydride in DMF gave DL-4,5-di-*O*-allyl-3,6-di-*O*-benzyl-1,2-*O*-cyclohexylidene-*myo*-inositol **(99)** in quantitative yield. The cyclohexylidene acetal was then removed by heating with 80% acetic acid for 4h to give DL-5,6-di-*O*-allyl-1,4-di-*O*-benzyl-*myo*-inositol **(100)** in 88% yield. At this stage, optical resolution was examined and it was found that treatment of DL-5,6-di-*O*-allyl-1,4-di-*O*-benzyl-*myo*-inositol with *l*-menthoxyacetyl chloride in pyridine at 0°C provided D-4,5-di-*O*-allyl-3,6-di-*O*-benzyl-1-*O*-menthoxyacetyl-*myo*-inositol **(101a)** in 39% yield [$R_f$ = 0.52, (ethyl acetate-hexane, 3:2)] and L-4,5-di-*O*-allyl-3,6-di-*O*-benzyl-1-*O*-menthoxyacetyl-*myo*-inositol **(101b)** in 43% yield [$R_f$ = 0.61, (ethyl acetate-hexane, 3:2)]. The keynote of the resolution was that the two diastereoisomers were efficiently separated by flash chromatography using ether-hexane, 1:2.

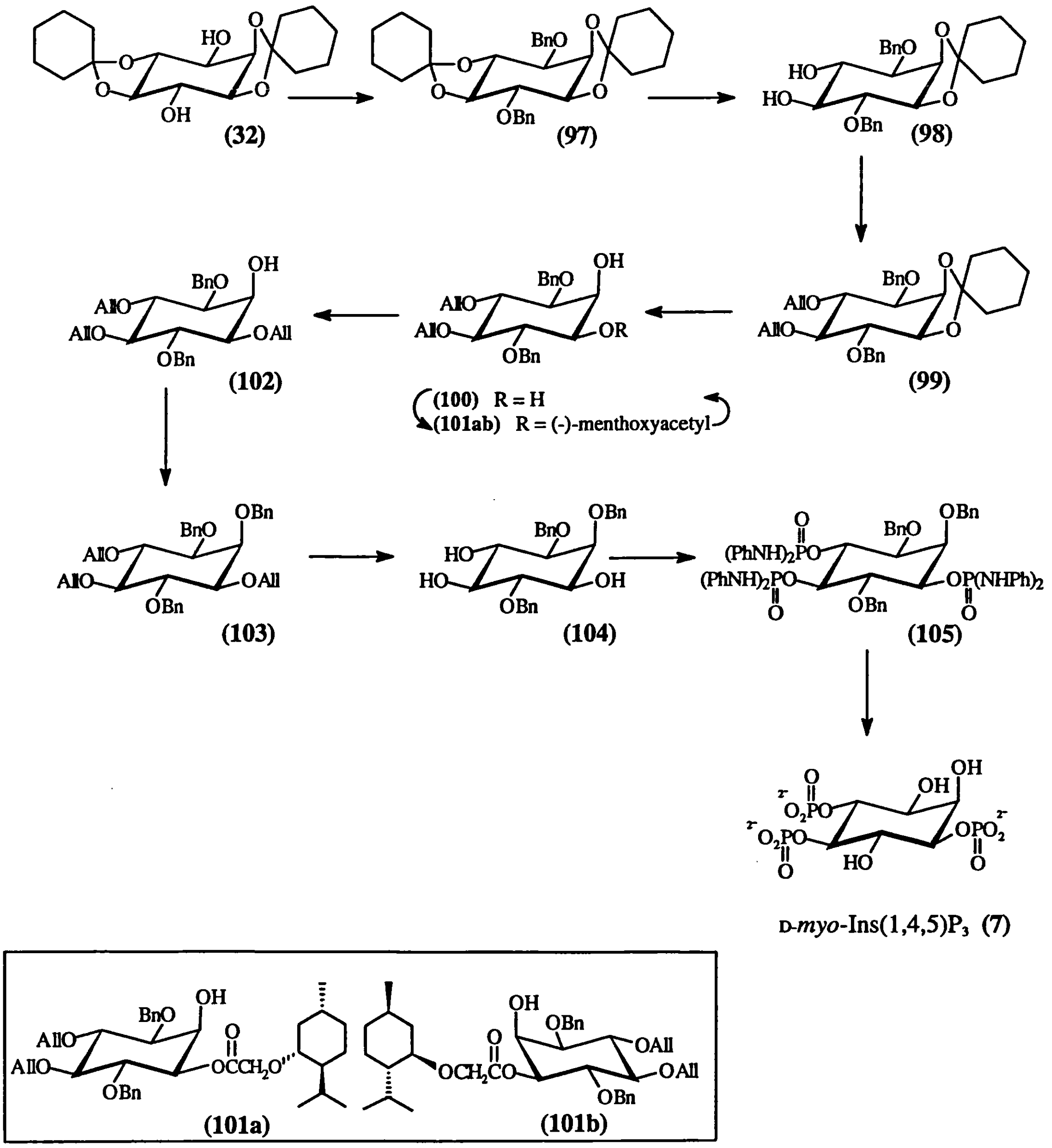



**Figure 39**

Recrystallisation of the reaction mixture from hexane in the presence of seed crystals also yielded the D-phosphorylation precursor. Each diastereoisomer was subjected to basic hydrolysis and transformed into their corresponding chiral 1,2-diols in quantitative yield. The absolute configuration was confirmed by transforming L-4,5-di-*O*-allyl-3,6-di-*O*-benzyl-1-menthoxyacetyl-*myo*-inositol into the known L-1,4,5,6-tetra-*O*-benzyl-*myo*-inositol, in five steps. D-4,5-Di-*O*-allyl-3,6-di-*O*-benzyl-*myo*-inositol was selectively allylated at the 1-position by treatment with allyl bromide in the presence of sodium hydroxide in benzene, under reflux for 80min to give D-1,4,5-tri-*O*-allyl-3,6-di-*O*-benzyl-*myo*-inositol **(102)** in 78% yield. The 2-hydroxyl position was benzylated as before to give the fully protected D-1,4,5-tri-*O*-allyl-2,3,6-tri-*O*-benzyl-*myo*-inositol **(103)**. The three allyl protective groups were eventually removed using Wilkinsons catalyst, [$(PPh_3)_3RhCl$] and (DABCO) in 90% aqueous ethanol for 8 h which provided the chiral precursor D-2,3,6-tri-*O*-benzyl-*myo*-inositol or, for lowest numbering, L-1,2,4-tri-*O*-benzyl-*myo*-inositol **(104)** in 59% yield. This compound has the necessary hydroxyl groups exposed so the penultimate stage of this synthesis was phosphorylation with dianilidophosphonic chloride in the presence of DMAP and pyridine to afford the totally protected L-1,2,4-tri-*O*-benzyl-3,5,6-tris-*O*-dianilinophospho-*myo*-inositol **(105)** in 41–60% yield. [395,396] Deprotection was accomplished in two steps by treatment of the totally protected derivative with excess isoamyl nitrite in a mixture of pyridine/acetic acid/acetic anhydride (1:1:1) followed by debenzylation at the 1-, 2- and 4-positions using 5% palladium on carbon under an atmosphere of hydrogen to furnish D-Ins(1,4,5)$P_3$ in 17% yield as well as a cyclic 4,5-pyrophosphate in a similar yield. The phosphorylation methods were subsequently improved by the introduction of *o*-xylene-*N,N*-diethylphosphoramidite, which was discussed in section 3.8.

### 3.9.2 Potter's Synthesis of Racemic Ins(1,4,5)$P_3$

Following Ozaki's synthesis of D-Ins(1,4,5)$P_3$, the second synthesis appeared a year later, by Potter and coworkers. [397] The advantage of this synthesis was the superior phosphorylation and deblocking methodology. However, a disadvantage of the synthesis was that the synthetic Ins(1,4,5)$P_3$ was racemic.

The versatile intermediate DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(37)** was synthesised in a three step reaction by treatment of *myo*-inositol with 2,2-dimethoxypropane in DMF and toluene-*p*-sulphonic acid as a catalyst. Benzoylation *in situ* with benzoyl chloride in pyridine furnished DL-3,6-di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol in 26% yield from *myo*-inositol. The two benzoates were deprotected using methanolic sodium hydroxide followed by neutralisation with carbon dioxide and solid extraction with dichloromethane to afford DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol in 94% yield. The diol was benzylated with benzyl bromide in DMF with sodium hydride as base to give DL-3,6-di-*O*-benzyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(106)** which was then subjected to mild acid treatment to remove the less stable *trans* diequatorial isopropylidene (55% yield) group, followed by allylation with allyl bromide and sodium hydride in DMF to give DL-4,5-di-*O*-allyl-3,6-di-*O*-benzyl-1,2-*O*-isopropylidene-*myo*-inositol **(107)**. The more stable *cis*-isopropylidene acetal was then removed under acidic conditions, methanol/1M HCl (9:1) in order to expose the 1,2-diol. The 1-position was selectively protected by tin-mediated allylation using allyl bromide in DMF to afford DL-1,4,5-tri-*O*-allyl-3,6-di-*O*-benzyl-*myo*-inositol **(108)** in 73% yield. The 2-position was benzylated with benzyl bromide in DMF, with sodium hydride as base to give the fully protected compound DL-1,4,5-tri-*O*-allyl-2,3,6-tri-*O*-benzyl-*myo*-inositol **(109)**. The three allyl groups were removed by one of two procedures. First, a one step deprotection using 10% palladium on carbon in the presence of toluene-*p*-sulphonic acid in an ethanol-water mixture, gave DL-1,2,4-tri-*O*-benzyl-*myo*-inositol **(110)** in 87% yield after refluxing for 4h. Second, isomerisation of the allyl moiety using freshly sublimed potassium *t*-butoxide in dry DMSO gave the DL-1,4,5-tri-*O*-*cis*-prop-1-enyl ether followed by acid treatment with acetone-1M HCl (9:1) at reflux for 30min to give DL-1,2,4-tri-*O*-benzyl-*myo*-inositol in 63% yield for the two step process.

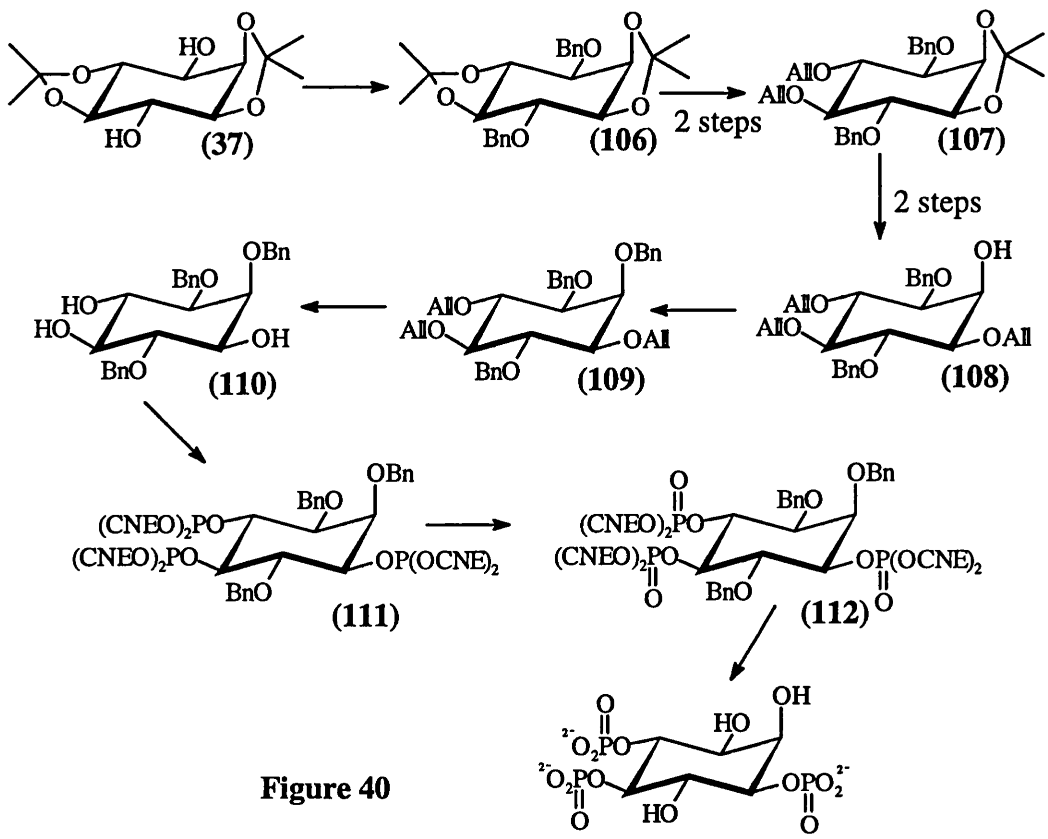



**Figure 40**

DL-Ins(1,4,5)$P_3$ **(113)**

The triol **(110)** was phosphorylated in a 3-step procedure using a P(III) reagent initially developed for DNA synthesis. [398] Thus, DL-1,2,4-tri-*O*-benzyl-*myo*-inositol was treated with diisopropylamino(2-cyanoethoxy)chlorophosphine **(91)** and *N,N*-diisopropylethylamine in dichloromethane to afford the trisphosphoramidite ($\delta_P$ = 150.46ppm) in *ca.* 90% yield. The reaction of the trisphosphoramidite with 2-cyanoethanol in the presence of 1*H*-tetrazole provided the trisphosphite intermediate **(111)**. This intermediate had 3 peaks in the phosphorus spectrum $\delta_P$ = 139.6ppm for P-1 and 140.2ppm, 141.0ppm for P-4 and P-5 (not assigned). At higher resolution there was a distinctive AB-coupling pattern for P-4 and P-5 $^5J_{PP}$ = 3.4Hz coupling which confirmed phosphitylation of the vicinal 4,5-diol. [399] The trisphosphite was oxidised with anhydrous *t*-butylhydroperoxide to afford the trisphosphate triesters $\delta_P$ ~ -3.4, in quantitative yield. The fully protected phosphate, 1,2,4-tri-*O*-benzyl-3,5,6-tris [di(cyanoethoxyphospho)]-*myo*-inositol **(112)** was deblocked in one step using sodium in liquid ammonia for 15min. The crude product was purified by ion exchange chromatography to provide racemic Ins(1,4,5)$P_3$ **(113)** and a small sample was further purified by HPLC using tritiated Ins(1,4,5)$P_3$ to permit detection.

### 3.9.3 Ley's Synthesis of D-Ins(1,4,5)$P_3$ from Benzene

There have been two different approaches for introducing protected hydroxyl groups with the correct stereochemistry to give Ins(1,4,5)$P_3$, using benzene as the starting material. The first was a racemic synthesis, which was slightly modified to provide a chiral synthetic route. Both the racemic and chiral synthesis of Ins(1,4,5)$P_3$ was carried out by Ley and coworkers. [400,401]

Benzene **(114)** was converted into *cis*-3,5-cyclohexadiene-1,2-diol **(115)** in a single step by microbial oxidation using *Pseudomonas putida.* This biotransformation was not possible by chemical means in a single step and provided a versatile building block for the synthesis of polyols. *cis*-3,5-Cyclohexadiene-1,2-diol is now commercially available [Fluka, (20%), 10ml, £50.30], albeit expensive. The *cis*-diol was envisaged as the C-2–C-3 functionality of the *myo*-inositol ring and was protected as the cyclic carbonate **(116)** by the reaction with sodium methoxide and dimethylcarbonate at room temperature. The 4,5-*trans*-diol moiety was introduced by epoxidation of the *meso*-carbonate **(116)** with *m*CPBA which gave the desired α-epoxide **(117)** in 47% yield together with the β-epoxide in 10% yield. The resolution was carried out at this stage by treatment of the racemic epoxide with a catalytic amount of tetrafluoroboric acid-diethyl ether in the presence of (*R*)-(+)-*sec*-phenethyl alcohol, to afford a (1:1) mixture of diastereoisomers in 67% yield, which were then separated by HPLC. The less polar diastereoisomer was utilised for the synthesis of D-Ins(1,4,5)$P_3$. The free hydroxyl group of compound **(118)** was benzylated under mild conditions by stirring benzyl bromide in the presence of silver (I) oxide in DMF to give compound **(119)**. In this way the C-4 and C-5 hydroxyl groups were protected in a similar manner so that deprotection later in the synthesis would expose the 1-, 4- and 5-hydroxyl groups in preparation for phosphorylation. The cyclic carbonate functionality was then hydrolysed with aqueous triethylamine in methanol, to furnish the diol, in which the hydroxyl groups at C-2 and C-3 were ideally placed for directed epoxidation across the olefin. Epoxidation with *m*CPBA (2 equivalents) gave the required β-epoxide **(120)** in 87% yield together with a small quantity (5%) of the α-epoxide, which were readily separated by column chromatography. The *cis*-diol at the C-2–C-3 positions was then protected as its isopropylidene acetal using 2,2-dimethoxypropane and a catalytic amount of camphorsulphonic acid in 89% yield. The ring opening of the β-epoxide **(120)** with the newly developed hydroxide equivalent sodium-2β-propoxy-5,5-dimethyl-1,3-dioxane **(121)** gave compound **(122)** as the major product in 58% yield. Catalytic hydrogenation of the 1-hydroxy derivative [5(*R*)]-D-4-*O*-benzyl-2,3-*O*-isopropylidene-6-*O*-[2-(5,5-dimethyl-1,3-dioxan-2-yl)ethyl]-5-*O*-(phenylethyl)-*myo*-inositol **(122)** exposed the 1-, 4- and 5-hydroxyl groups which were ready for phosphorylation. The reaction of three equivalents of *n*-butyl lithium and tetrabenzylpyrophosphate in THF and *N,N*-diisopropylethylamine furnished the *myo*-inositol-1,4,5-trisphosphate derivative **(123)** in 67% yield. Deprotection by hydrogenation, followed by acid treatment with 80% aqueous trifluoroacetic acid gave the crude product which was purified by HPLC to give D-Ins(1,4,5)$P_3$ **(7)** in 88% yield after deprotection.

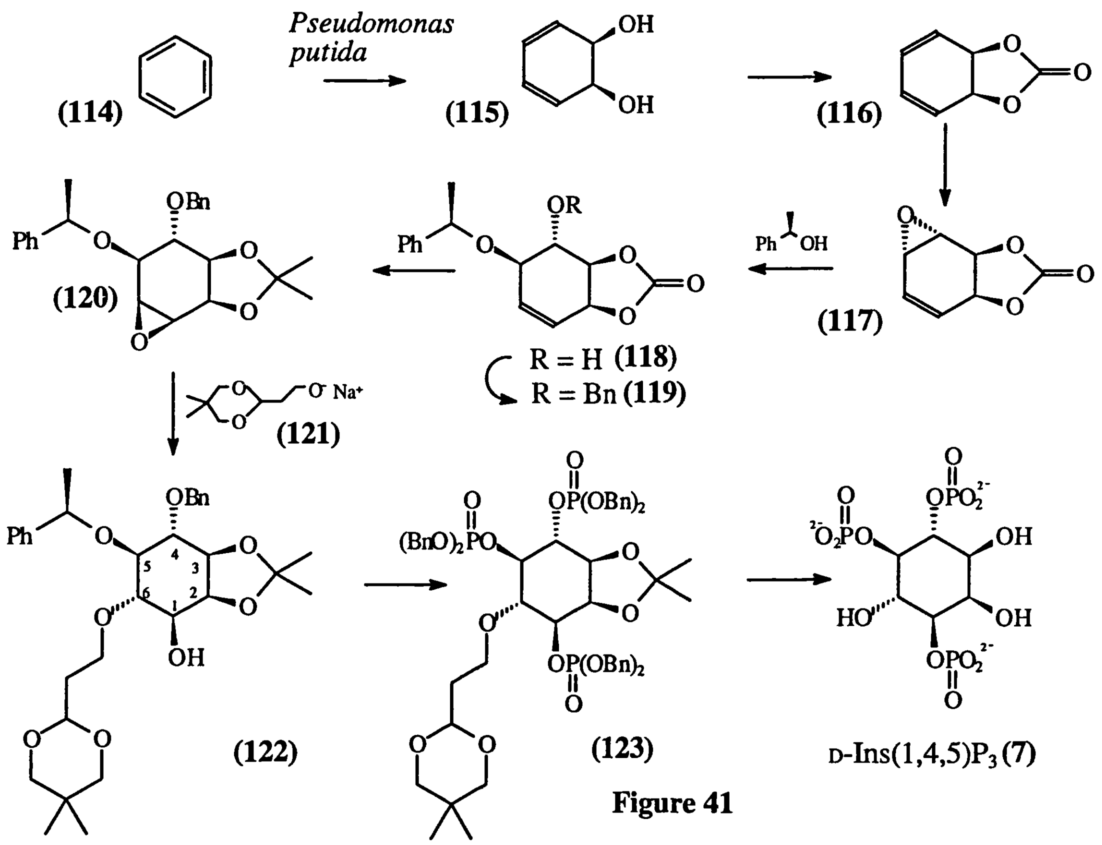



**Figure 41**

### 3.9.4 Carless's Synthesis of Racemic Ins(1,4,5)$P_3$ from Benzene

The second approach towards the synthesis of *myo*-inositol phosphate derivatives relied on the *cis*-hydroxylation of the all-equatorial conduritol B, which was prepared from benzene in the following manner (Figure 42). [402]

Benzene was subjected to Birch reduction, *trans*-hydroxylation, acetylation and dehydrobromination in an overall yield of 40% to give the *trans*-diol **(124)**. This compound was then protected as its di-(2-methoxyethoxymethyl) ether using 2-methoxyethoxymethyl chloride (MEM-Cl) and *N,N*-diisopropylethylamine as base, in 91% yield. The 1- and 4-hydroxyl groups were introduced into the 6-membered ring by treatment of the diene with singlet oxygen in a [2+4] cycloaddition to give the endoperoxide which yielded the conduritol F derivative **(125)** by stereospecific reduction with thiourea/methanol in 70% yield. The hydroxyl group at C-1 of the conduritol F derivative was then inverted by an oxidation-reduction transformation. Oxidation with pyridinium chlorochromate (PCC, 1.5 equivalents) gave two separable hydroxyenones, **(126)** in 50% and another isomer in 20% yield respectively. Reduction of **(126)** in the presence of sodium borohydride/cerium trichloride gave the symmetrical conduritol B derivative **(127)**. A more efficient alternative was found by oxidising both 1- and 4-hydroxyl groups with PCC (3 equivalents), to give the enedione, which did not undergo tautomerisation to the corresponding aromatic system, but was reduced with sodium borohydride/cerium trichloride to give the conduritol B derivative in 70% yield. Benzylation of the conduritol B derivative with sodium hydride and benzyl bromide was achieved in 95% yield. The two remaining *cis*-hydroxyl groups were introduced using osmium tetroxide/*N*-methylmorpholine-*N*-oxide in 96% yield to give the *myo*-inositol derivative **(128)**. Selective hydroxyl protection at the 3-position using MEM-Cl in 60% yield followed by benzylation at the 2-position, gave DL-1,2,4-tri-*O*-benzyl-3,5,6-tri-*O*-(2-methoxyethoxymethyl)-*myo*-inositol **(129)**. The three MEM-protective groups were removed using 6M HCl, in THF at 20°C to give DL-1,2,4-tri-*O*-benzyl-*myo*-inositol **(110)**. The triol was phosphorylated with tetrabenzyl pyrophosphate and sodium hydride to give the totally protected intermediate in 47% yield. Catalytic hydrogenation, using palladium on carbon gave DL-Ins(1,4,5)$P_3$ **(113)**.

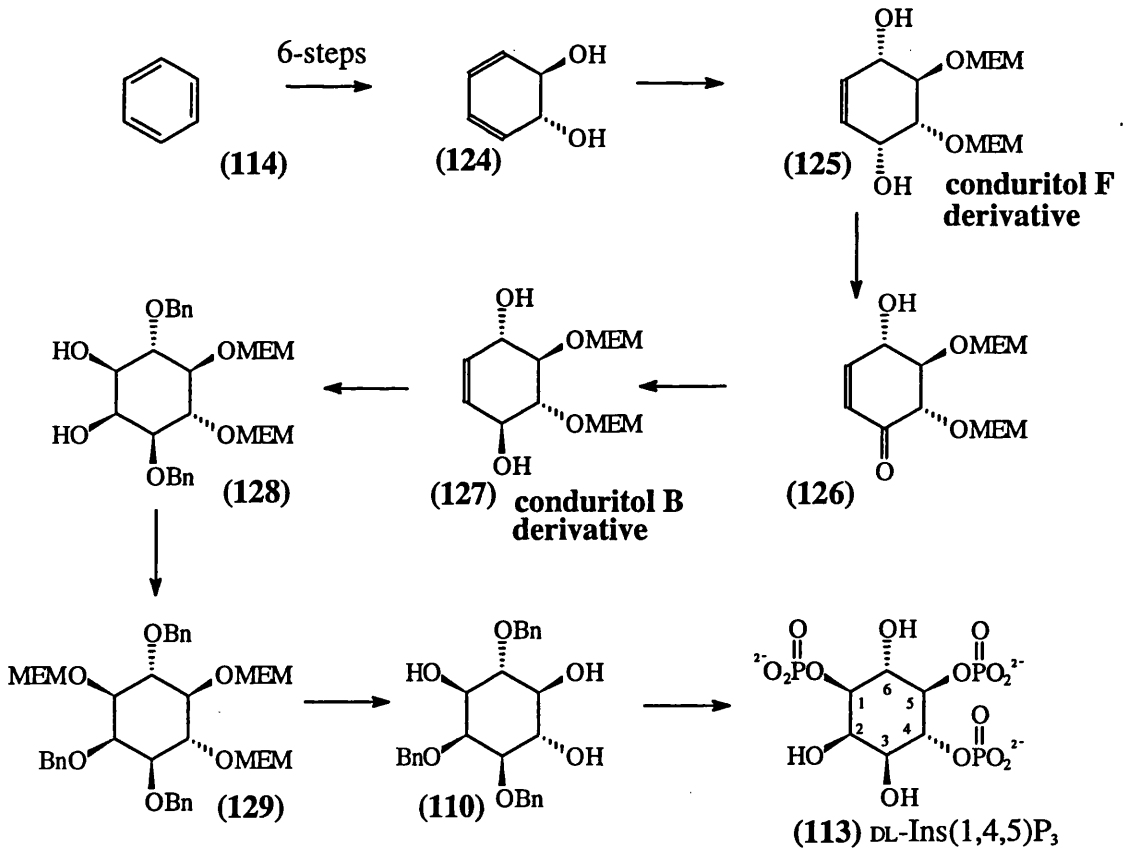



**Figure 42**

### 3.9.5 Falck's Synthesis of D-Ins(1,4,5)$P_3$ from (-)-Quinic acid

D-(-)-Quinic acid **(130)** is a readily available, reasonably inexpensive [100g, £46.30 (Aldrich 1993/94)] chiral starting material, which possesses 4 hydroxyl groups and a carboxyl group. However, satisfactory solutions must be addressed for several outstanding issues for the synthesis of D-Ins(1,4,5)$P_3$ from D-(-)-quinic acid. First, differentiation of the hydroxyl groups, second, stereospecific oxidation of both methylene groups, corresponding to C-4 and C-6 of Ins(1,4,5)$P_3$ and finally the removal of the carboxyl group at the C-5 position of Ins(1,4,5)$P_3$. The realisation of these objectives together with the inversion of the hydroxyl group at C-1 of the *myo*-inositol derivative has led to the efficient chiral synthesis of D-Ins(1,4,5)$P_3$ from D-(-)-quinic acid. [403]

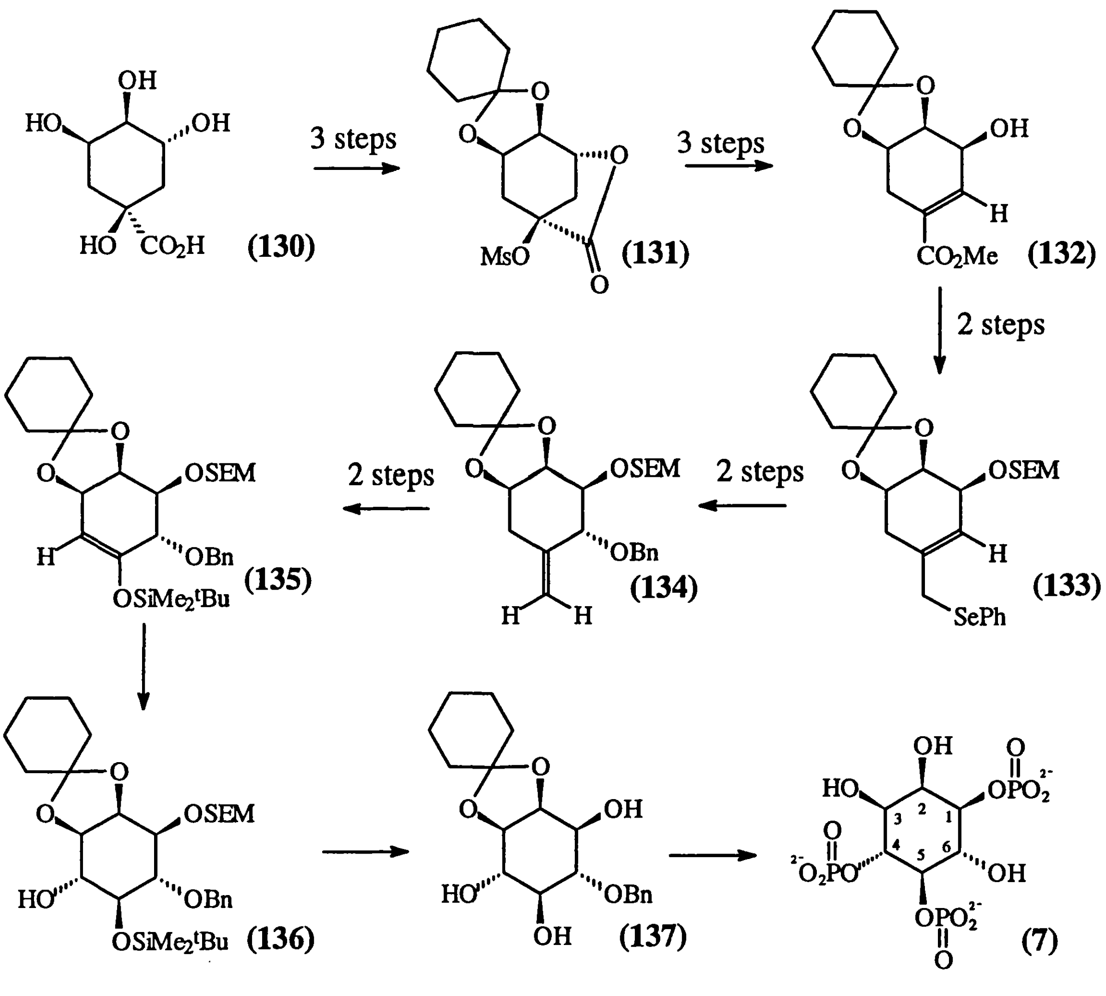



**Figure 43**

The first step was to adjust the stereochemistry at C-1 to give a *myo*-inositol derivative. This was accomplished by modifying a literature procedure. (-)-Quinic acid was converted into the lactone by a two-step one pot lactonisation and cyclohexylidenation using cyclohexanone and Amberlite IR-120 acid resin, followed by mesylation of the tertiary alcohol to give compound **(131)**. The lactone was methanolysed and the C-1 hydroxyl (*myo*-inositol numbering) was oxidised with PCC and triethylamine-induced mesylate elimination provided the enone. The enone was reduced to the enol by delivery to the less hindered β-face using sodium borohydride, to provide compound **(132)**.

The C-1 hydroxyl group now had the correct equatorial orientation and this was protected as the β-trimethylsilylethoxymethyl (SEM) ether. The ester function was reduced to the primary alcohol using diisobutylaluminium hydride (DIBAL-H) at -78° C followed by selenation of the primary alcohol with *N*-(phenylseleno)phthalimide in the presence of tributylphosphine, to give **(133)**. A stereoselective [2,3]-sigmatropic rearrangement of the allylic selenoxide followed by benzylation with benzyl bromide and potassium hydride gave **(134)** as the only product.

The methylene group at C-4 was functionalised by ozonolysis of **(134)** and the resulting ketone was converted into the silylenol ether **(135)** using *t*-butyldimethylsilyl (TBDMS) trifluoromethane sulphonate with complete regiospecificity. Hydroboration with borane/THF from the β-face followed by alkaline hydrogen peroxide oxidation provided the fully hydroxylated *myo*-inositol derivative **(136)**. The TBDMS group at the 5-position was easily removed with fluoride whereas the SEM protective group at the 1-position was only removed under more drastic conditions, using tetrabutylammonium fluoride, in hexamethylphosphoric triamide (HMPA) at 100°C. Finally, phosphorylation of this chiral intermediate, L-4-*O*-benzyl-1,2-*O*-cyclohexylidene-*myo*-inositol **(137)** with tetrabenzylpyrophosphate followed by hydrogenolysis and acid treatment, gave D-Ins(1,4,5)$P_3$ **(7)** in 62% yield for the deprotection steps.

### 3.9.6 Ballou's Synthesis of D-Ins(1,4,5)$P_3$ from D-Pinitol

Another strategy that has been adopted for the synthesis of D-Ins(1,4,5)$P_3$ used D-(+)-3-*O*-methyl-*chiro*-inositol, **(138)** or D-pinitol, as starting material. [404] D-Pinitol reaches 20% of the dry weight of the cold water extract of sugar pine (*Pinus lambertiana Dougl*) stump wood. [405]

D-(+)-Pinitol was demethylated by treatment with hydroiodic acid in acetic acid to give D-(+)-*chiro*-inositol. The reaction of D-(+)-*chiro*-inositol **(27)** with dimethoxycyclohexane in the presence of an acid catalyst resulted in the formation of D-1,2:5,6-di-*O*-cyclohexylidene-*chiro*-inositol **(139)**. The hydroxyl groups at C-3 and C-4 were benzylated using neat benzyl chloride and solid potassium hydroxide as base and an 18-crown-6 phase transfer catalyst to give D-3,4-di-*O*-benzyl-1,2:5,6-di-*O*-cyclohexylidene-*chiro*-inositol. The 3,4-di-*O*-benzyl derivative was treated with 80% acetic acid at 100°C for 2h to provide the 1,2,5,6-tetrol. This tetrol was benzoylated selectively at the 1-, 2- and 5-positions using benzoyl chloride at 5°C for 2 days then 23°C for 1 day and 80°C for 18h to give 1,2,5-tri-*O*-benzoyl-3,4-di-*O*-benzyl-D-*chiro*-inositol **(140)**. The axial hydroxyl group at C-6 (D-*chiro*-inositol numbering) was inverted *via* the trifluoromethanesulphonate ester using sodium acetate in acetic acid to give a 3:1 mixture (70% yield) of L-1,2,4-tri-*O*-benzoyl-5,6-tri-*O*-benzyl-*myo*-inositol **(143)** and L-1,3,4-tri-*O*-benzoyl-5,6-tri-*O*-benzyl-*myo*-inositol **(142)**. The reaction proceeded *via* a benzyloxonium ion which preferentially opens to give the axial benzoyl group. The intermediate **(143)** was the one required for the synthesis of D-Ins(1,4,5)$P_3$. Thus, L-1,2,4-tri-*O*-benzoyl-*myo*-inositol **(144)** was obtained by hydrogenolysis of the benzyl groups at positions 5- and 6- (*myo*-inositol numbering).

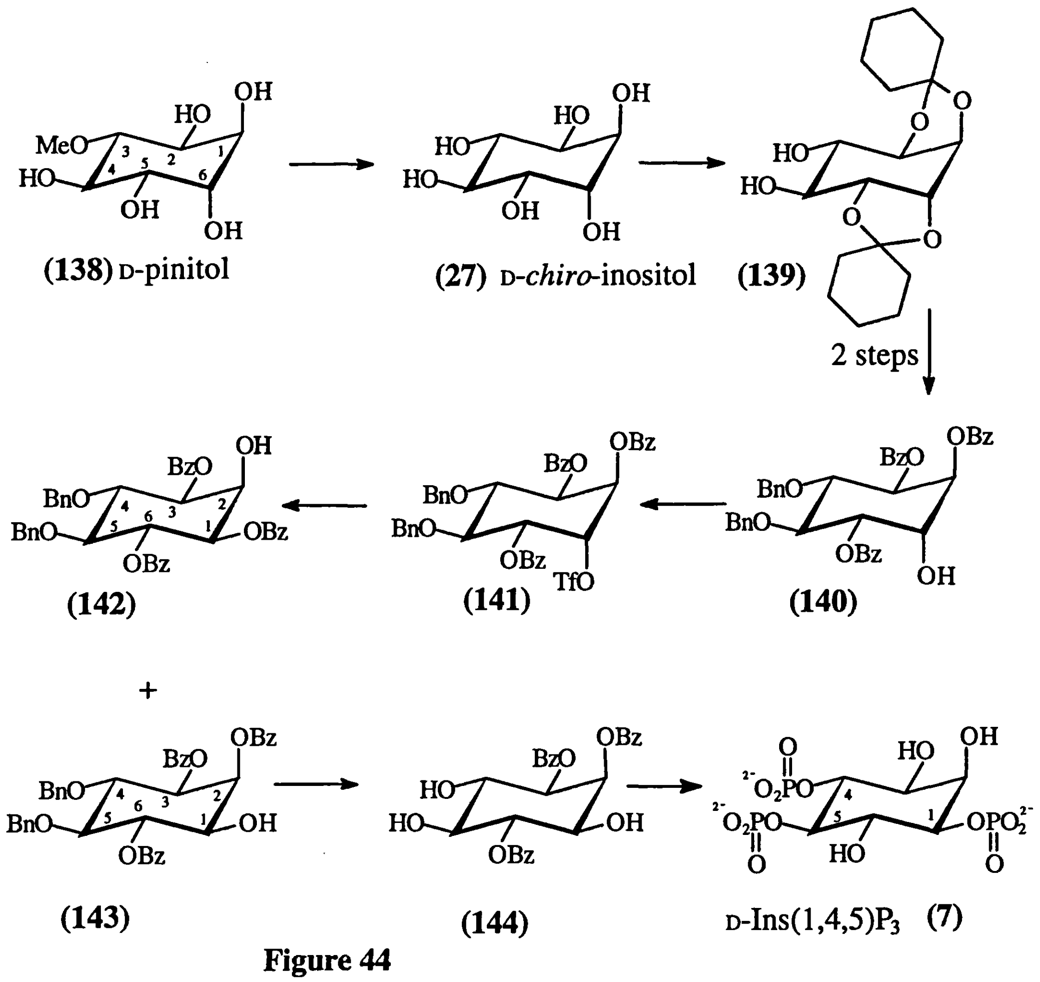



**Figure 44**

Phosphorylation was accomplished by the P(III) method, using bis(benzyloxy) diisopropylaminophosphine and 1*H*-tetrazole in dichloromethane. Oxidation with *m*CPBA gave the totally protected 1,4,5-trisphosphate in 94% yield. Hydrogenolysis with 5% palladium on charcoal, followed by basic hydrolysis to saponify the benzoyl esters, provided D-Ins(1,4,5)$P_3$ as its cyclohexylamine salt in 87% yield.

There are four main reviews on the chemistry of inositol phosphates. However, they are all several years old. [406-409]

# CHAPTER FOUR

# A Review Of The Structure-Activity Relationships At The Ins(1,4,5)$P_3$ Receptor And The Enzymes 3-Kinase And 5-Phosphatase

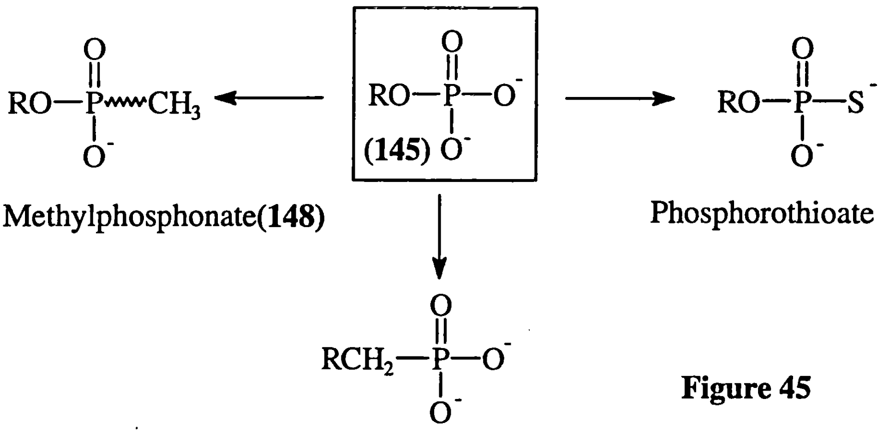
## 4.1 Introduction

The second messenger Ins(1,4,5)$P_3$ has been synthesised by many groups and by several different approaches, some of which were discussed in Chapter 3. Now that all the problems concerning the introduction of phosphate groups into the *myo*-inositol ring have been overcome, the next stage is to modify the structure of Ins(1,4,5)$P_3$ in order to provide analogues which may have a different effect at the Ins(1,4,5)$P_3$ receptor. Ins(1,4,5)$P_3$ binds to the N-terminus of its receptor resulting in the release of $Ca^{2+}$ ions from intracellular stores and thereafter, the second messenger must be destroyed. There are two avenues for the deactivation of Ins(1,4,5)$P_3$ which have already been discussed in detail.

This chapter will mainly deal with small molecule Ins(1,4,5)$P_3$ receptor agonists together with 3-kinase and 5-phosphatase inhibitors, which were designed, based on the Ins(1,4,5)$P_3$ structure and which are modified at the hydroxyl and phosphate groups. No small molecule Ins(1,4,5)$P_3$ receptor antagonist has been presented to date. However, a number of partial agonists have been synthesised and evaluated, and further modification will hopefully develop a lead to provide a small molecule, full antagonist. The number of analogues that have been synthesised over the last few years has increased, therefore only a selected number of chemical schemes will be presented. The emphasis will be placed on the chemical modification of the Ins(1,4,5)$P_3$ structure together with the pharmacology of the molecule so as to provide a structure-activity relationship, and show how these modifications affect interaction with the receptor as well as with the enzymes 3-kinase and 5-phosphatase. Finally, the two Ins(1,4,5)$P_3$ receptor antagonists, heparin and the polyanion decavanadate will be discussed.

## 4.2 Modification of the Ins(1,4,5)$P_3$ structure

Modification of the Ins(1,4,5)$P_3$ structure falls into two divisions. First, modulation of the phosphate group (Figure 45) and second, adjustment of the hydroxyl moiety in some manner. Phosphate groups have been modified by replacing one of the oxygen atoms of the phosphate with a sulphur atom so producing a phosphorothioate analogue **(146)**. Generally, the phosphorothioate group is more resistant to enzyme hydrolysis by phosphatases therefore providing a longer half-life and a different biological activity from the natural substrate. The phosphorothioate group is larger than the phosphate moiety, thus increasing steric demand. The nucleophilic nature of the sulphur moiety also provides a point of attachment for environmentally-sensitive reporter groups, such as fluorescent and spin labels. The negative charge on the phosphorothioate is present on the sulphur atom rather than the oxygen atom. Phosphorothioate groups are more acidic than phosphates and generally less stable because they are prone to air oxidation. Therefore, care must be taken when handling compounds containing phosphorothioate groups.



Methylphosphonate(**148**)

Phosphorothioate (**146**)

Methylenephosphonate (**147**)

**Figure 45**

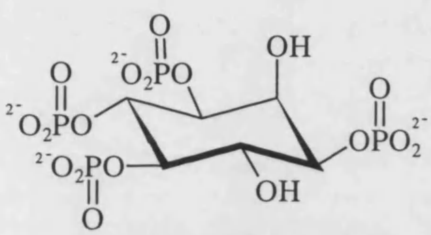
Other modifications of the phosphate moiety include the introduction of a methyl or methylene group onto the phosphorus atom. If a methylene group bridging the attached to the *myo*-inositol ring carbon and the phosphorus atom a methylenephosphonate (**147**) is produced. On the other hand if a methyl group is attached to the phosphorus atom then a methyl phosphonate (**148**) is produced. Most of the useful phosphate-modified analogues have been derived from phosphorothioate substitution.

Modulation of the *myo*-inositol hydroxyl moiety has been carried out in order to provide analogues which were not metabolised by the two Ins(1,4,5)$P_3$-deactivating enzymes, 3-kinase and/or 5-phosphatase. The hydroxyl group has been replaced by simple deletion (replacing -OH by -H) in order to provide deoxy-derivatives with no potential H-bonding at that position. Fluoro, or difluoro analogues, have also replaced the hydroxyl and hydroxyl/hydrogen functions respectively, on the inositol ring. Fluoro analogues are H-bond acceptors only, and are a useful hydroxyl replacement to probe for this type of interaction. The fluorine substituent is isoelectronic with the hydroxyl group but is more hydrophobic. Alkylation with methyl iodide provides a methyl ether, where no H-bonding at that site can take place, and may deliver a hydrophobic interaction with the outcome of possible antagonistic activity. The hydroxyl groups may be inverted at the 2-, 3- and 6-positions in order to give analogues of Ins(1,4,5)$P_3$. Not all the hydroxyl positions have been inverted, however, and such future work will provide important information on the structure-activity relationships. Finally, replacement of the hydroxyl function *via* the azide followed by hydrogenolysis has provided the more basic amino-moiety, with possible interaction with the neighbouring phosphate group in which the amino function presumably exists as $NH_3^+$.

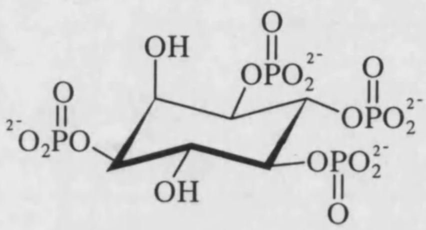
### 4.3 *myo*-Inositol Tetrakisphosphates and Phosphorothioates

Not many *myo*-inositol tetrakisphosphate analogues have been synthesised, and therefore data on their interaction with the Ins(1,4,5)$P_3$ receptor, Ins(1,3,4,5)$P_4$ receptor, or with the two enzymes 5-phosphatase or 3-kinase are scarce.

#### 4.3.1 D-Ins(1,3,4,5)$P_4$ and L-Ins(1,3,4,5)$P_4$



D-Ins(1,3,4,5)$P_4$ **(149)**



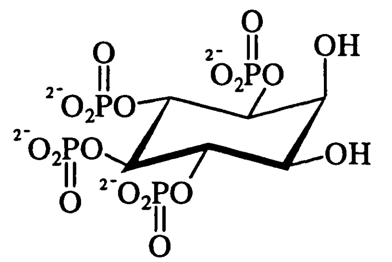
L-Ins(1,3,4,5)$P_4$ **(150)**

**Figure 46**

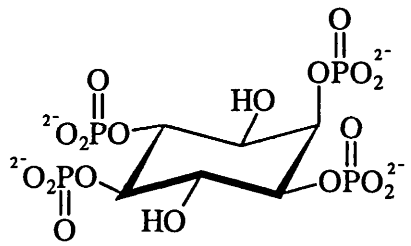
D-*myo*-Inositol 1,3,4,5-tetrakisphosphate **(149)**, Ins(1,3,4,5)$P_4$, mobilised all the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores in SH-SY5Y human neuroblastoma cells with an $EC_{50}$ value of 2.05μM [218] compared with 0.14μM for Ins(1,4,5)$P_3$. However, L-*myo*-inositol 1,3,4,5-tetrakisphosphate **(150)**, L-Ins(1,3,4,5)$P_4$, failed to mobilise $Ca^{2+}$ at concentrations up to 100μM. Pig cerebellar membranes contain both Ins(1,4,5)$P_3$ and Ins(1,3,4,5)$P_4$ binding sites. In experiments using these membranes, both L-Ins(1,3,4,5)$P_4$ and L-Ins(1,4,5)$P_3$ were greater than 1000-fold weaker in displacing specific [$^3$H]Ins(1,4,5)$P_3$ at the Ins(1,4,5)$P_3$ receptor. Whereas D-Ins(1,3,4,5)$P_4$ ($IC_{50}$ = 762nM) was only 40-fold weaker than D-Ins(1,4,5)$P_3$ ($IC_{50}$ = 20.7nM). In contrast, the Ins(1,3,4,5)$P_4$ receptor showed poor stereoselectivity. Both D-Ins(1,3,4,5)$P_4$ and L-Ins(1,3,4,5)$P_4$ showed biphasic displacement of [$^3$H]Ins(1,3,4,5)$P_4$, with high (2.1nM) and low (918nM) affinity sites for D-Ins(1,3,4,5)$P_4$ and less than 10-fold weaker than L-Ins(1,3,4,5)$P_4$.

### 4.3.2 Ins(3,4,5,6)$P_4$

In two articles, Hirata and coworkers [410,411] have demonstrated a role for *myo*-inositol 3,4,5,6-tetrakisphosphate **(151)** Ins(3,4,5,6)$P_4$, in gating $Ca^{2+}$ influx. NIH/3T3 fibroblasts possess a $Ca^{2+}$ influx pathway and when these cells were held at -40mV, followed by the application of Ins(3,4,5,6)$P_4$, an elevation of $[Ca^{2+}]_i$ was observed lasting for 2-3min. [410] This influx was not observed in the absence of extracellular $Ca^{2+}$. However, when Ins(3,4,5,6)$P_4$ was applied to *ras*-transformed NIH/3T3 fibroblasts (DT) cells held at -40mV, an initial elevation of $[Ca^{2+}]_i$ was observed followed by a substantial increase in the presence of extracellular $Ca^{2+}$. Indeed, in *ras*-transformed NIH/3T3 fibroblasts, the application of Ins(1,3,4,5)$P_4$ or Ins(1,3,4,6)$P_4$ mimicked the bradykinin-induced increase of $[Ca^{2+}]_i$ with little or very little specificity between the three. [$Ca^{2+}$ influx mediated by Ins(1,3,4,5)$P_4$ and Ins(1,3,4,6)$P_4$ has been discussed in 2.7.7]. In conclusion, Ins(3,4,5,6)$P_4$ in conjunction with other tetrakisphosphates may provide a mechanism to maintain $[Ca^{2+}]_i$ at a certain level during cytosolic $Ca^{2+}$ oscillations following agonist stimulation in *ras*-transformed NIH/3T3 fibroblast cells.



Ins(3,4,5,6)$P_4$ **(151)**



DL-Ins(1,2,4,5)$P_4$ **(152)**

**Figure 47**

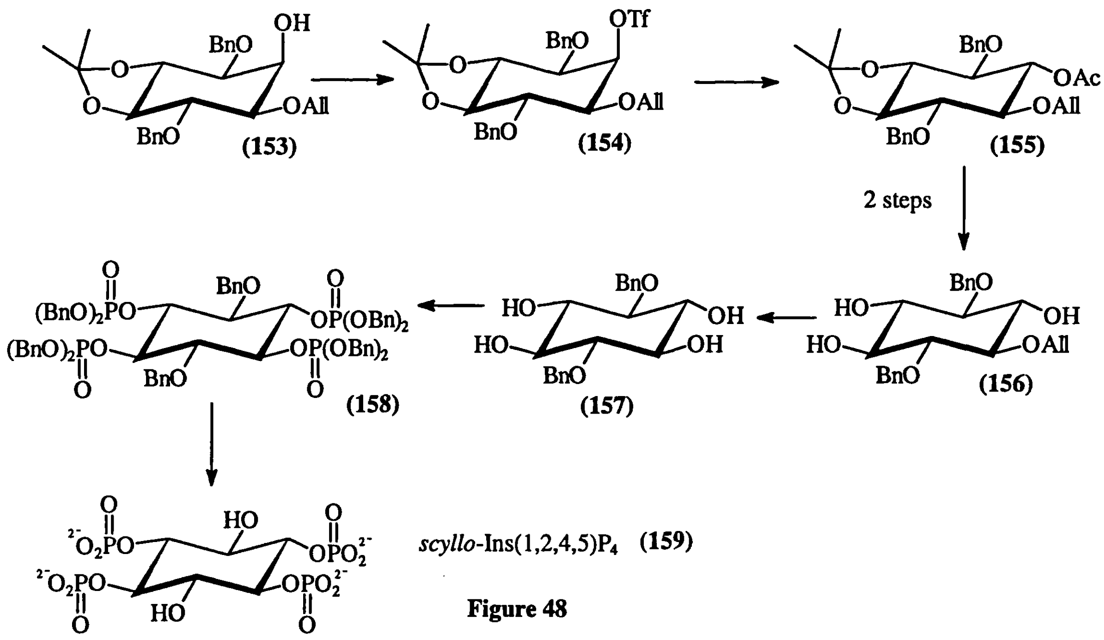
### 4.3.3 DL-Ins(1,2,4,5)$P_4$

Ozaki and coworkers were the second group to provide biological data for racemic Ins(1,2,4,5)$P_4$. DL-1,4-Di-*O*-benzyl-*myo*-inositol was phosphitylated with bis (benzyloxy)diisopropylaminophosphine followed by oxidation with *m*CPBA. Column chromatography gave the fully protected phosphorylated compound in 96% yield. Hydrogenolysis using palladium on carbon, in methanol-water mixture gave racemic Ins(1,2,4,5)$P_4$ **(152)** in 77% yield. [412]

In the purified rat cerebellum, it was shown that DL-Ins(1,2,4,5)$P_4$ **(152)** inhibited the binding of [$^3$H]Ins(1,4,5)$P_3$ with a half maximal concentration of 11.3nM, compared with 7.1nM for Ins(1,4,5)$P_3$. The $EC_{50}$ for $Ca^{2+}$-release from nonmitochondrial pools of permeabilised rat basophilic leukemic (RBL) cells for Ins(1,4,5)$P_3$ was 0.5μM, whereas for DL-Ins(1,2,4,5)$P_4$ it was 1.2μM. Assuming that L-Ins(1,2,4,5)$P_4$ does not contribute to $Ca^{2+}$-release, then D-Ins(1,2,4,5)$P_4$ is nearly as potent as Ins(1,4,5)$P_3$ and substitution with a hydrophilic phosphate group at the 2-position did not appear to significantly decrease binding at the Ins(1,4,5)$P_3$ receptor. DL-Ins(1,2,4,5)$P_4$ was found to be a potent inhibitor of 5-phosphatase from erythrocyte ghosts. It is difficult to speculate whether one or both enantiomers play an important part in the inhibition process. A Dixon plot showed that DL-Ins(1,2,4,5)$P_4$ behaved as a competitive inhibitor of 5-phosphatase with a $K_i$ value of 1.4μM, whereas the $K_m$ value for Ins(1,4,5)$P_3$ was 16.8μM. The compound was not recognised by 3-kinase from rat brain cytosol.

DL-Ins(1,2,4,5)$P_4$ has also been synthesised by Meek and coworkers [393] and also Carless and coworkers. [402] No biological data have been presented by these groups.

### 4.3.4 *scyllo*-Ins(1,2,4,5)$P_4$




*scyllo*-Ins(1,2,4,5)$P_4$ **(159)**

**Figure 48**

*scyllo*-Inositol 1,2,4,5-tetrakisphosphate **(159)**, *scyllo*-Ins(1,2,4,5)$P_4$, was synthesised from the intermediate DL-1-*O*-allyl-3,6-di-*O*-benzyl-4,5-*O*-isopropylidene-*myo*-inositol **(153** in Figure 48). First, the unsubstituted axial 2-hydroxyl position was blocked with the excellent leaving group, trifluoromethane sulphonate **(154)** using trifluoromethane sulphonic anhydride in pyridine. The 2-position was set-up for $S_N2$ substitution, which was achieved using caesium acetate in DMF at room temperature to provide the equatorial *scyllo*-derivative **(155)**. The isopropylidene group was removed under acidic conditions, followed by saponification of the acetate moiety to give **(156)**. The allyl group of compound **(156)** was isomerised to the *cis*-prop-1-enyl ether using Bu$^t$OK/DMSO followed by acid hydrolysis to provide the symmetrical derivative 1,4-di-*O*-benzyl-*scyllo*-inositol **(157)**. Phosphitylation of the four hydroxyl groups with bis(benzyloxy)diisopropylaminophosphine and 1*H*-tetrazole followed by oxidation, provided the totally protected *scyllo*-derivative **(158)**. Chemical reduction using sodium in liquid ammonia removed all ten of the benzyl protective groups to give the symmetrical *scyllo*-Ins(1,2,4,5)$P_4$ **(159)** in 41% yield. [413]

*scyllo*-Ins(1,2,4,5)$P_4$ was found to be a potent full agonist at the Ins(1,4,5)$P_3$ receptor from saponin-permeabilised SH-SY5Y cells with an $EC_{50}$ value for $Ca^{2+}$-release of 82.56nM. [*cf* Ins(1,4,5)$P_3$ $EC_{50}$ for $Ca^{2+}$-release was 52nM]. *scyllo*-Ins(1,2,4,5)$P_4$ displaced [$^3$H]Ins(1,4,5)$P_3$ from specific sites on bovine adrenal cortex membranes with an $IC_{50}$ of 14.37nM, and with a $K_i$ value of 10.82nM. *scyllo*-Ins(1,2,4,5)$P_4$ was found to be a substrate for 5-phosphatase from human erythrocyte ghosts (HEG) $K_i$ value of 4.3μM and also a substrate for 3-kinase from crude rat brain homogenate supernatant (CRBHS) with a $K_i$ value of 25.1μM. [414]

### 4.3.5 Ins(1,3,4,5)$P_4$-3S

Two approaches to the synthesis of *myo*-inositol 3-phosphorothioate-1,4,5-trisphosphate, Ins(1,3,4,5)$P_4$-3S have been published. The first to be published was a synthesis of the racemate by Liu and Potter (in Figure 49), [415] to give **(168)** and the second was a chiral synthesis by Kozikowski and coworkers (in Figure 50) [416] to give **(176)**.

The first synthesis was carried out by using *myo*-inositol as the starting material. DL-1,2:4,5-Di-*O*-isopropylidene-*myo*-inositol **(37)** was prepared in a 3-step procedure described by Gigg and coworkers. [344] Selective allylation at the 3-position was achieved using allyl bromide in the presence of barium oxide/barium hydroxide in 63% yield. Benzylation at the 6-position using benzyl chloride and sodium hydride in DMF in quantitative yield provided the totally protected compound DL-3-*O*-allyl-6-*O*-benzyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(160)**. The isopropylidene groups were removed under acidic conditions and a *p*-methoxybenzyl group was introduced selectively at the 1-position *via* the stannylene acetal, in the presence of caesium fluoride and *p*-methoxybenzyl chloride in 74% yield, to give compound **(161)**. The 4,5-*O*-isopropylidene acetal was reintroduced to give **(162)** and the free 2-hydroxyl was subsequently benzylated to give the fully protected derivative. The 4,5-*O*-isopropylidene acetal and the *p*-methoxybenzyl group were removed using 1M HCl under reflux, to give DL-1-*O*-allyl-2,4-di-*O*-benzyl-*myo*-inositol **(163)** in 90% yield. The allyl group was isomerised to the *cis*-prop-1-enyl ether **(164)** and the exposed hydroxyl groups at the 1-, 4- and 5-positions were phosphitylated using bis(benzyloxy)diisopropylaminophosphine in the presence of 1*H*-tetrazole, and then oxidised to provide the fully protected trisphosphate **(165)** in 80% yield. The *cis*-prop-1-enyl ether was removed using trifluoroacetic acid in order to expose the 3-hydroxyl **(166)** and was set-up for phosphitylation at the 3-position followed by sulphoxidation using sulphur in pyridine to provide the totally protected 3-phosphorothioate-1,4,5-trisphosphate **(167)**. The decabenzyl derivative **(167)** was deprotected in one step using sodium in liquid ammonia followed by ion exchange chromatography on Q-Sepharose, eluting with a gradient of triethylammonium bicarbonate (TEAB) buffer (0$\rightarrow$1M) to give DL-*myo*-inositol 3-phosphorothioate-1,4,5-trisphosphate **(168)** in 68% yield.

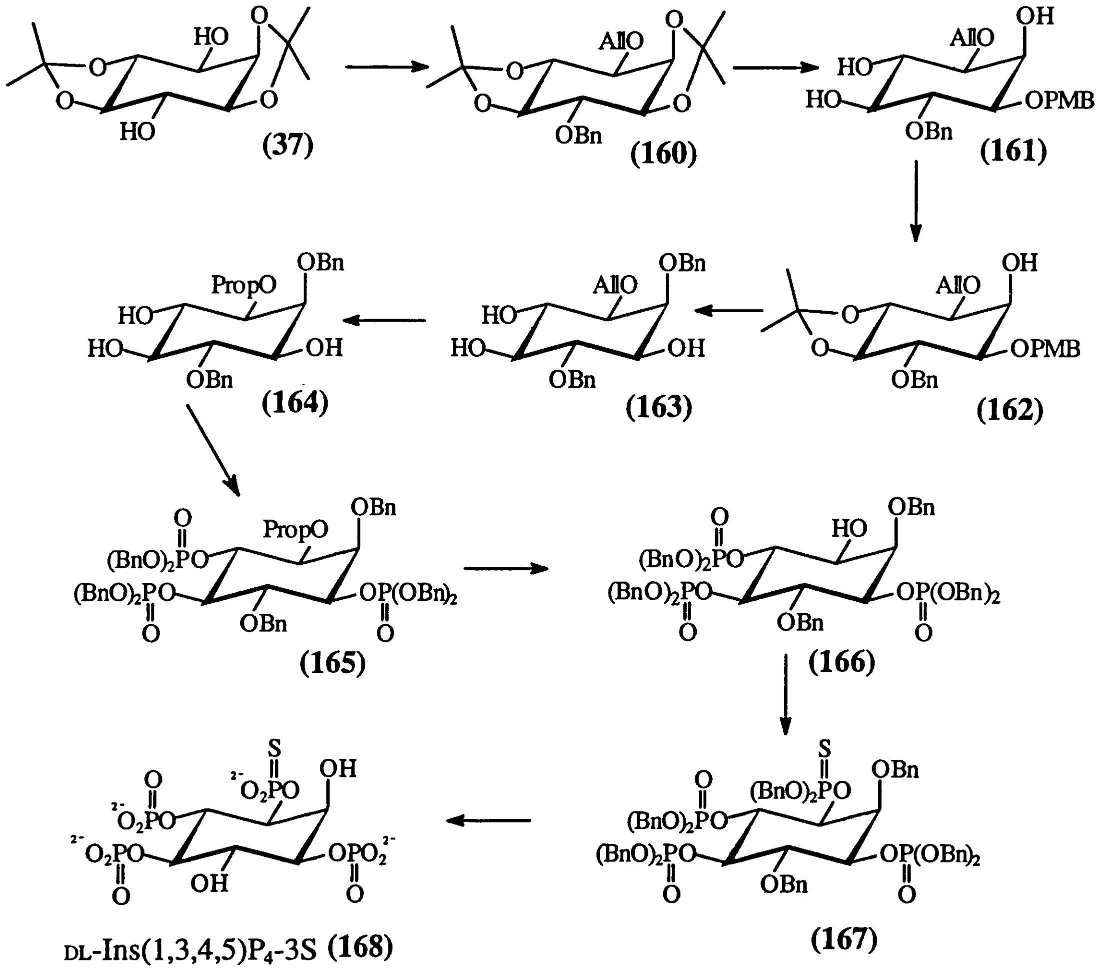



**Figure 49**

It has been reported [417] that Ins(1,3,4,5)$P_4$ acts as a full agonist for $Ca^{2+}$-release in the presence of the 3-phosphatase inhibitor, phytic acid, with an $EC_{50}$ value of 2.5μM. DL-Ins(1,3,4,5)$P_4$-3S **(168)** was also a weak, albeit full agonist for $Ca^{2+}$-release in permeabilised neuroblastoma cells with an $EC_{50}$ of 4.7μM which is similar to Ins(1,3,4,5)$P_4$. [417] DL-Ins(1,3,4,5)$P_4$-3S specifically displaced [$^3$H]Ins(1,4,5)$P_3$ from specific sites on bovine adrenal cortex membranes with an $IC_{50}$ of 889nM, whereas Ins(1,4,5)$P_3$ had an $IC_{50}$ value of 4.4nM. For Ins(1,3,4,5)$P_4$ the $IC_{50}$ value was 152nM. The analogue also appeared to be a substrate for 5-phosphatase from HEG and inhibited dephosphorylation of [$^3$H]Ins(1,4,5)$P_3$ with a $K_i$ value of 0.14μM. The 3-phosphorothioate substitution will provide an important probe to investigate the second messenger function of Ins(1,3,4,5)$P_4$ which is resistant to 3-phosphatase action.

A different approach, starting from L-quebrachitol has been reported by Kozikowski and coworkers. [416] L-Quebrachitol **(169)** was protected as the 3,4:5,6-di-*O*-isopropylidene derivative **(170)** using 2-methoxypropene in the presence of an acid catalyst in DMF at 70°C for 5h. Mesylation at the axial 1-position followed by demethylation at the 2-position and concomitant removal of the 3,4:5,6-di-*O*-isopropylidene groups with boron tribromide, exposed the hydroxyl groups which were subsequently protected at the 3, 4, 5 and 6-positions as the di-*O*-isopropylidene derivative to give L-1-*O*-methanesulphonyl-2,3:5,6-di-*O*-isopropylidene-*chiro*-inositol **(171)** which could be separated from L-3,4:5,6-di-*O*-isopropylidene-*chiro*-inositol derivative by crystallisation. Benzylation at the 4-hydroxyl position followed by demethanesulphonylation with lithium aluminium hydride, exposed the 1-hydroxyl derivative, which was inverted to give the 3-hydroxy-*myo*-inositol derivative **(172)** by Swern oxidation followed by lithium borohydride reduction. The exposed hydroxyl group at the D-3-position was protected as a *p*-methoxybenzyl ether and the *trans*-4,5-*O*-isopropylidene group was cleaved using a catalytic amount of acetyl chloride in methanol. The 4,5-diol was benzoylated with benzoyl chloride in pyridine and the *cis*-1,2-*O*-isopropylidene group was hydrolysed using concentrated HCl in methanol at room temperature to give the diol **(173)**. Selective benzoylation at the equatorial 1-hydroxyl (89% yield) followed by protection at the 2-position as a (benzyloxy)methyl ether gave compound **(174)**. The benzoate groups at the 1-, 4- and 5-positions were removed by alkaline methanolysis and the exposed hydroxyl groups were phosphorylated using tetrabenzyl pyrophosphate and sodium hydride to give the fully protected 1,4,5-trisphosphate **(175)**. Exposure of this compound to wet DDQ removed the *p*-methoxybenzyl ether at the 3-position in 87% yield which then allowed phosphitylation followed by sulphoxidation with phenylacetyl disulphide. Chemical reduction of all the protective groups using sodium in liquid ammonia gave D-Ins(1,3,4,5)$P_4$-3S **(176)** which was characterised as its triethylammonium salt.

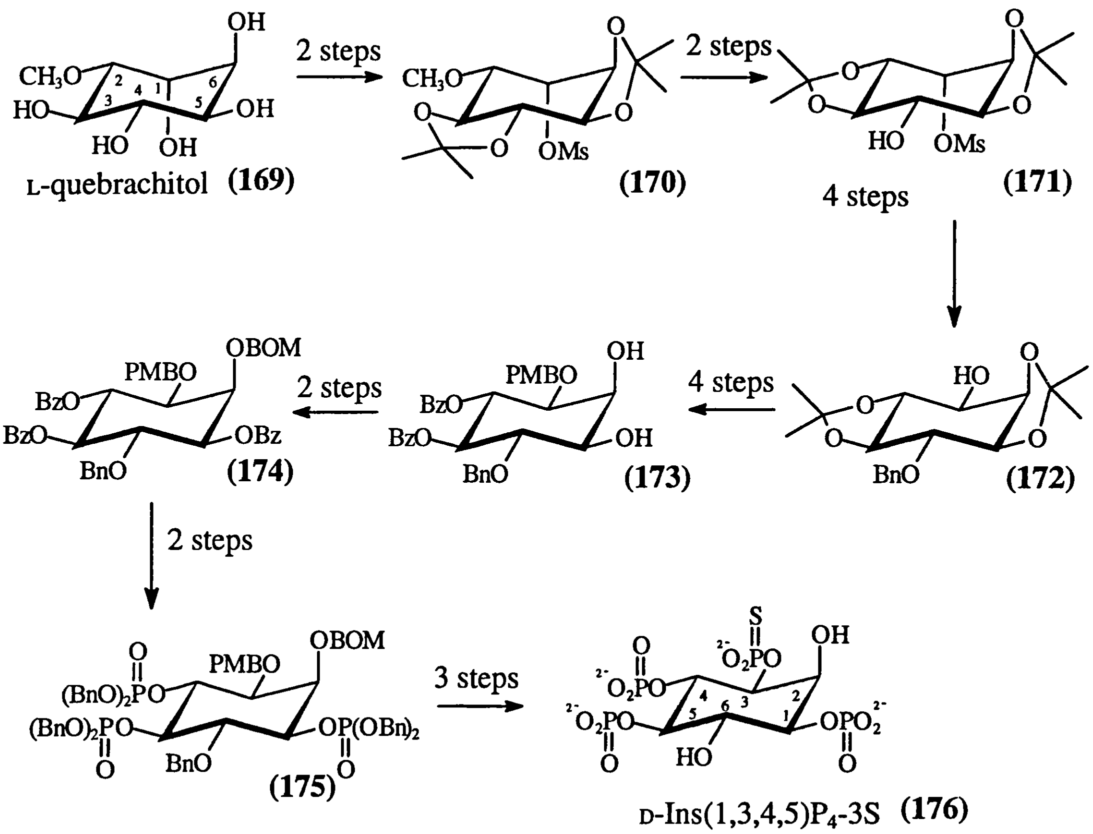



**Figure 50**

D-Ins(1,3,4,5)$P_4$-3S displaced [$^3$H]Ins(1,4,5)$P_3$ from specific sites on bovine adrenal cortex membranes with an $IC_{50}$ of 279.9nM. It was a full, albeit weak agonist with an $EC_{50}$ value of 2.53μM. The synthesis of another analogue, DL-Ins(1,3,4,5)$P_4$-5S has also been published [392] but, no biological data have been described and will not be discussed any further.

### 4.4 Inositol Trisphosphate Analogues

#### 4.4.1 L-*chiro*-Ins(2,3,5)$P_3$ and L-*chiro*-Ins(1,4,6)$P_3$

The starting material for the synthesis of L-*chiro*-inositol 2,3,5-trisphosphate, [L-*chiro*-Ins(2,3,5)$P_3$] was L-quebrachitol (**169** in Figure 51). L-Quebrachitol [418,419] was demethylated using concentrated HI to provide L-*chiro*-inositol (**28**) in 80% yield. The treatment of L-*chiro*-inositol in the presence of four equivalents of dibutyltin oxide and a five-fold excess of tetrabutylammonium iodide in acetonitrile provided the crystalline L-2,3,5-tri-*O*-benzyl-*chiro*-inositol (**177**) in 32% yield. The remaining three hydroxyl groups were benzoylated to give L-1,4,6-tri-*O*-benzoyl-2,3,5-tri-*O*-benzyl-*chiro*-inositol (**178**) in quantitative yield. The benzyl groups were removed by hydrogenation using a palladium catalyst in order to provide the phosphorylation precursor, L-1,4,6-tri-*O*-benzoyl-*chiro*-inositol (94% yield) **(179)**. The cheap, commercially available phosphitylating reagent, diethoxychlorophosphine, was used in the presence of dry *N,N*-diisopropylethylamine and acetonitrile to phosphitylate the 2-, 3- and 5-hydroxyl groups followed by oxidation to the P(V) intermediate **(180)** with *t*-butylhydroperoxide in 73% yield. The six ethyl groups of the protected phosphate were removed using bromotrimethylsilane in dichloromethane, followed by treating the evaporated residue with water in order to provide the free phosphate. The benzoyl groups were hydrolysed with 0.5M NaOH, followed by treatment with Dowex 50($H^+$) resin and final purification using ion exchange chromatography on DEAE Sephadex A-25 to give L-*chiro*-Ins(2,3,5)$P_3$ in 86.5% yield. A synthesis of DL-*chiro*-Ins(2,3,5)$P_3$ has been carried out by Carless and Busia. [420]

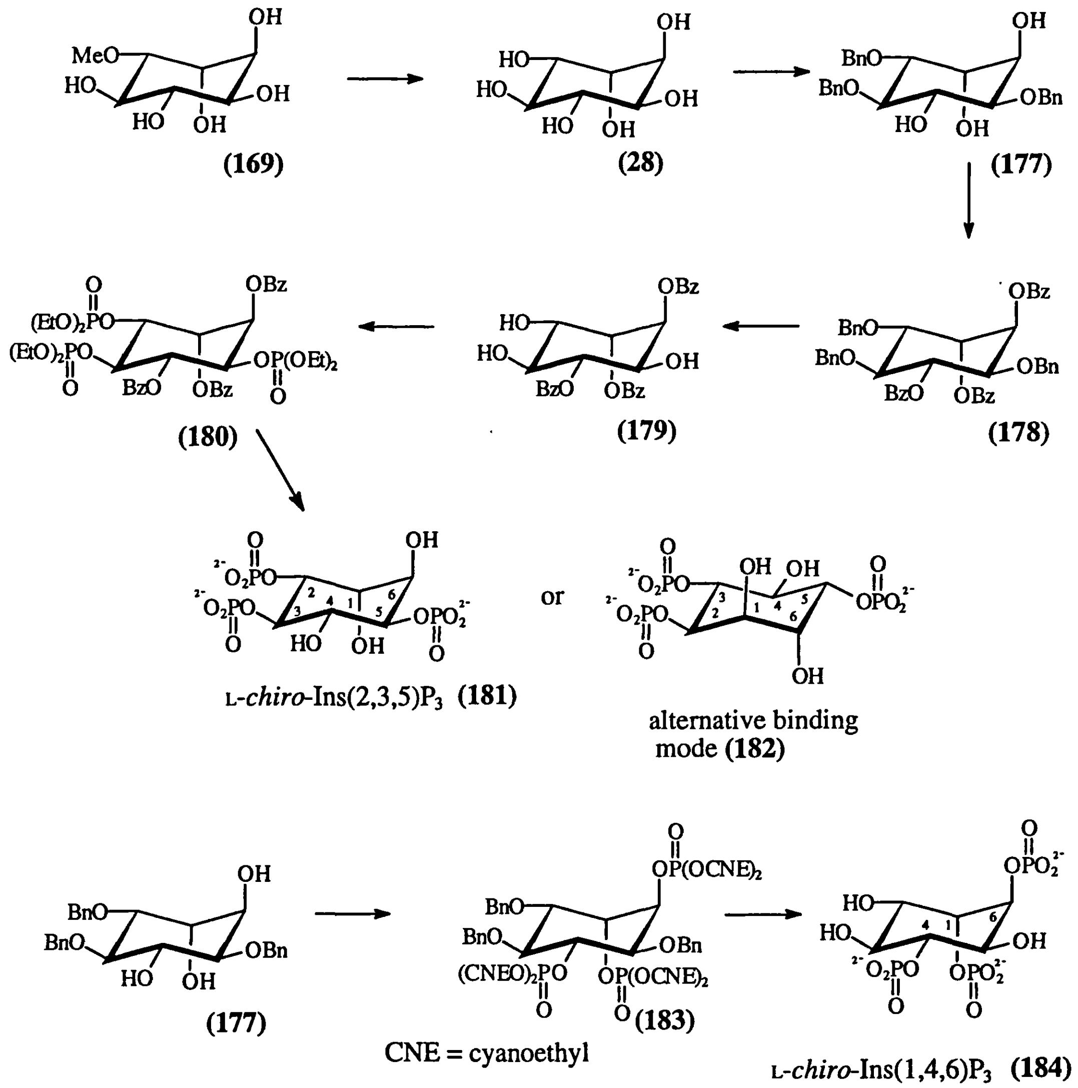



**Figure 51**

If the equatorial 3-hydroxyl group of Ins(1,4,5)$P_3$ is inverted to provide an axial hydroxyl group then L-*chiro*-Ins(2,3,5)$P_3$ is produced. It was envisaged that the inverted hydroxyl moiety may provide an inhibitor of the 3-kinase enzyme. L-*chiro*-Ins(2,3,5)$P_3$ was found to be a potent agonist for the release of $Ca^{2+}$ from permeabilised SH-SY5Y cells with an $EC_{50}$ of 1.4μM. It displaced [$^3$H]Ins(1,4,5)$P_3$ from specific sites on bovine adrenal cortex membranes with an $IC_{50}$ of 60.4nM. The analogue was also a potent inhibitor of 3-kinase ($K_i$ of 7.1μM, then re-evaluated to a $K_i$ of 0.97μM). [421,422] Significantly, L-*chiro*-Ins(2,3,5)$P_3$ was a potent inhibitor of 5-phosphatase ($K_i$ of 7.7μM). Two possible explanations for 5-phosphatase inhibition by L-*chiro*-Ins(2,3,5)$P_3$ were given. First, the conformation of L-*chiro*-Ins(2,3,5)$P_3$ in solution and/or bound to the 5-phosphatase may be sufficiently different from Ins(1,4,5)$P_3$ to interfere with the catalytic mechanism of 5-phosphatase and bind to it in a manner similar to Ins(1,4,5)$P_3$ as shown in (**181** in Figure 51). The inhibition of 5-phosphatase by L-*chiro*-Ins(2,3,5)$P_3$ may result from an inverted and rotated mode of binding **(182)**, where the ring pucker, the vicinal 4,5-bisphosphate and the 3-hydroxyl group, [represented as the 4-hydroxyl group in L-*chiro*-Ins(2,3,5)$P_3$] may result. The 5-phosphate group of L-*chiro*-Ins(2,3,5)$P_3$ represented an equatorial 2-phosphate of Ins(1,4,5)$P_3$. Furthermore, the two axial groups of L-*chiro*-Ins(2,3,5)$P_3$ at the 1- and 6-positions, now mimic the 6- and 1-positions of Ins(1,4,5)$P_3$.

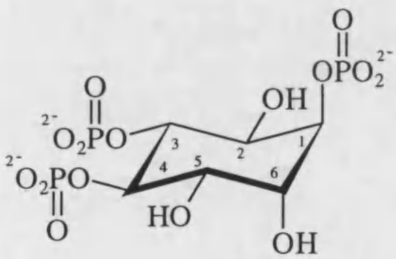
The analogue L-*chiro*-Ins(1,4,6)$P_3$ (**184** in Figure 51) was also synthesised from L-quebrachitol *via* the precursor L-2,3,5-tri-*O*-benzyl-*chiro*-inositol **(177)**. [423,424] The hydroxyl groups at the 1-, 4- and 6-positions were phosphitylated with bis(2-cyanoethoxy)diisopropylaminophosphine and 1*H*-tetrazole in dry dichloromethane, followed by oxidation to P(V) with *t*-butylhydroperoxide in 75% yield after chromatography. Deprotection of the totally protected compound **(183)** with sodium in liquid ammonia provided L-*chiro*-Ins(1,4,6)$P_3$ **(184)** in 75% yield after ion exchange chromatography on DEAE Sephadex A-25. This compound did not show any $Ca^{2+}$-releasing properties from permeabilised SH-SY5Y cells, but it was a moderately potent inhibitor of Ins(1,4,5)$P_3$ 5-phosphatase with a $K_i$ value of 44μM. This compound did not interact with 3-kinase.

In another system, L-*chiro*-Ins(1,4,6)$P_3$ competitively inhibited the specific 5-phosphate dephosphorylation of Ins(1,4,5)$P_3$ and Ins(1,3,4,5)$P_4$ from skeletal muscle with $K_i$ values of 6.35 and 1.76μM respectively. [425]

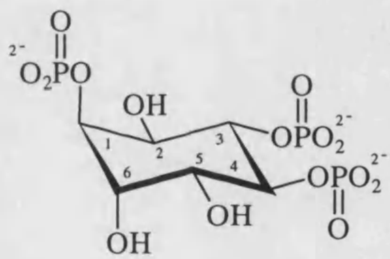
### 4.4.2 D- And L-*chiro*-Ins(1,3,4)$P_3$, D- and L-Ins(2,4,5)$P_3$

The synthesis of D-*chiro*-Ins(1,3,4)$P_3$ **(185)** and L-*chiro*-Ins(1,3,4)$P_3$ **(186)** started with the chiral intermediates D- and L-1,2,5-tri-*O*-benzoyl-3,4-di-*O*-benzyl-*chiro*-inositol, obtained from D-*chiro*-inositol and L-*chiro*-inositol respectively, as described in section 3.9.6. The two benzyl groups from each of the two enantiomers were removed quantitatively by hydrogenation. Phosphitylation of the resulting triol, using bis(benzyloxy)diisopropylaminophosphine followed by oxidation with *m*CPBA, gave the totally protected 1,3,4-tris(dibenzyloxyphospho)-2,5,6-tri-*O*-benzoyl-*chiro*-inositol in 84% yield for the D-enantiomer and 77% for the L-enantiomer. The individual enantiomers were deprotected by hydrogenation over a palladium on carbon catalyst followed by saponification with sodium hydroxide. The cyclohexylamine salt of the D-enantiomer was crystallised from water and 2-propanol in 71% yield. [426]

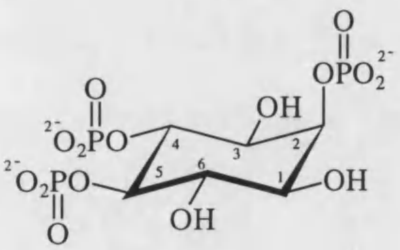
The analogue D-*chiro*-Ins(1,3,4)$P_3$ was tested for $Ca^{2+}$-release in permeabilised rat basophilic leukemic (RBL) cells using fluo-3 as the $Ca^{2+}$ indicator. The $EC_{50}$ value was found to be 4.2μM, compared with an $EC_{50}$ value of 0.17μM for Ins(1,4,5)$P_3$. This value was very similar to D-Ins(2,4,5)$P_3$ and indicated that if the 1-position of D-Ins(2,4,5)$P_3$ was inverted to give an axial hydroxyl and thus D-*chiro*-Ins(1,3,4)$P_3$, then no effect on $Ca^{2+}$-release would be observed.



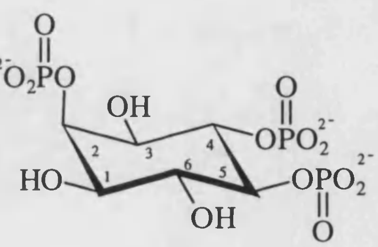
D-*chiro*-Ins(1,3,4)$P_3$ **(185)**



L-*chiro*-Ins(1,3,4)$P_3$ **(186)**



D-*myo*-Ins(2,4,5)$P_3$ **(187)**



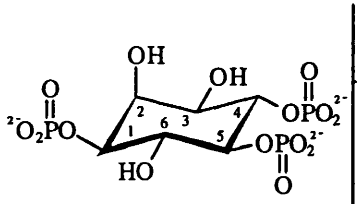
L-*myo*-Ins(2,4,5)$P_3$ **(188)**

**Figure 52**

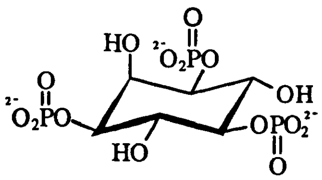
The analogues D-Ins(2,4,5)$P_3$ **(187)** and L-Ins(2,4,5)$P_3$ **(188)** were synthesised from D- and L-*chiro*-inositol. The *chiro*-inositols were transformed into 1,3,4-tri-*O*-benzoyl-5,6-di-*O*-benzyl-*myo*-inositol, which were minor products resulting from inversion, (see section 3.9.6). The benzyl groups were removed by hydrogenation in quantitative yield to provide 1,3,4-tri-*O*-benzoyl-L-*myo*-inositol, from D-*chiro*-inositol and 1,3,4-tri-*O*-benzoyl-D-*myo*-inositol, from L-*chiro*-inositol. The individual triols were phosphorylated and deprotected as for the D- and L-*chiro*-Ins(1,3,4)$P_3$ in order to provide D- and L-Ins(2,4,5)$P_3$. [426]

The $Ca^{2+}$-releasing properties of D-Ins(2,4,5)$P_3$ in permeabilised RBL cells was found to be 4.3μM. The $Ca^{2+}$-releasing activity of L-Ins(2,4,5)$P_3$ was 25-fold less than for the D-isomer, having an $EC_{50}$ value of 110μM, albeit lower than L-Ins(1,4,5)$P_3$ with an $EC_{50}$ value of more than 2000μM, *i.e.* no real activity. The $Ca^{2+}$-releasing activity of L-Ins(2,4,5)$P_3$ and L-*chiro*-Ins(1,3,4)$P_3$ may be due to the isomers having an axial phosphate which may be flexible enough to allow an induced fit at the active site of the receptor by ring distortion or flipping. [426]

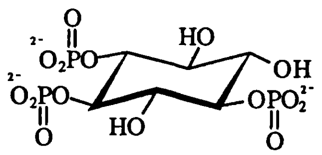
### 4.4.3 L-Ins(1,4,5)$P_3$, Ins(1,3,5)$P_3$ and DL-*scyllo*-Ins(1,2,4)$P_3$



L-*myo*-Ins(1,4,5)$P_3$ **(189)**



Ins(1,3,5)$P_3$ **(190)**



DL-*scyllo*-Ins(1,2,4)$P_3$ **(191)**

**Figure 53**

L-Ins(1,4,5)$P_3$ **(189)** was synthesised from the known chiral intermediate D-1,2,4-tri-*O*-benzyl-*myo*-inositol, originally prepared by Gigg and coworkers. [383] The analogue was found to be a weak inhibitor of Ins(1,4,5)$P_3$ 5-phosphatase from bovine aorta with a $K_i$ value of 39μM. [427] It was poorly recognised by 3-kinase from crude bovine aortic cytosol, while the $EC_{50}$ value was 220μM for $Ca^{2+}$-mobilisation in permeabilised bovine aortic smooth muscle cells. In another study, [422] L-Ins(1,4,5)$P_3$ inhibited Ins(1,4,5)$P_3$ 5-phosphatase from HEG with a $K_i$ value of 15.3μM. L-Ins(1,4,5)$P_3$ interacted modestly with 3-kinase from CRBHS with a $K_i$ value of 40.3μ M and had an $EC_{50}$ value >>30μM.

The *meso*-compound Ins(1,3,5)$P_3$ **(190)** was also tested by Polokoff and coworkers [427] and was shown to be a competitive inhibitor of 5-phosphatase, with a $K_i$ value of 45μM. It was not effectively recognised by 3-kinase, with a $K_i$ value of 1687μM and did not release $Ca^{2+}$ effectively, with an $EC_{50}$ value of 630μM.

The first *scyllo*-inositol phosphate analogue to be synthesised was DL-*scyllo*-inositol 1,2,4-trisphosphate **(191)**, DL-*scyllo*-Ins(1,2,4)$P_3$. This analogue differs from Ins(1,4,5)$P_3$ in that the axial 2-position has been inverted to provide an equatorial hydroxyl function. It was synthesised from DL-1-*O*-allyl-3,6-di-*O*-benzyl-4,5-*O*-isopropylidene-*myo*-inositol **(153)**. The 2-position was blocked with a triflate **(154)** and then inverted with caesium acetate/DMF at room temperature in an $S_N2$ manner to provide the suitable *scyllo*-inositol derivative **(155)**. [428] The 4,5-*O*-isopropylidene acetal was removed by acid hydrolysis followed by saponification of the acetate group to provide the racemic compound DL-2-*O*-allyl-1,4-di-*O*-benzyl-*scyllo*-inositol **(156)**. This was phosphitylated with bis(benzyloxy)diisopropylaminophosphine and 1*H*-tetrazole followed by oxidation, to give the trisphosphate in 58% yield. Deprotection of all protective groups using sodium in liquid ammonia provided DL-*scyllo*-Ins(1,2,4)$P_3$ **(191)** which was purified by ion exchange chromatography. *scyllo*-Ins(1,2,4)$P_3$ potently induced $Ca^{2+}$-mobilisation in saponin permeabilised SH-SY5Y cells with an $EC_{50}$ value of 171nM. The analogue was also tested for its ability to interact with 5-phosphatase from HEG and 3-kinase from CRBHS. It was found that the analogue was a moderate 5-phosphatase inhibitor with a $K_i$ value of 24.2μM but was susceptible to 3-kinase activity, and was rapidly metabolised by SH-SY5Y cells. [414]

### 4.4.4 3-Modified Ins(1,4,5)$P_3$-3R Analogues: R = Carboxyl, Methyl, Ethyl and *n*-Propyl

All four racemic compounds, DL-Ins(1,4,5)$P_3$-3-*O*-methylenecarboxylate **(195)**, DL-3-*O*-methyl-Ins(1,4,5)$P_3$ **(196)**, DL-3-*O*-ethyl-Ins(1,4,5)$P_3$ **(197)** and DL-3-*O*-*n*-propyl-Ins(1,4,5)$P_3$ **(198)** were synthesised by to Liu and Potter, [429,430] using the versatile intermediate DL-3-*O*-allyl-2,6-di-*O*-benzyl-4,5-*O*-isopropylidene-1-*O*-*p*-methoxy benzyl-*myo*-inositol **(192)**. This intermediate was also used to synthesise *myo*-inositol 1,4,5-trisphosphate-3-phosphorothioate **(168)** and shall be the starting point for the synthesis of the 3-modified derivatives (Figure 54).

First, DL-Ins(1,4,5)$P_3$-3-*O*-methylenecarboxylate **(195)**, was synthesised by removing the 4,5-*O*-isopropylidene group and the 1-*O*-*p*-methoxybenzyl ether by treatment with acid to give DL-1-*O*-allyl-2,6-di-*O*-benzyl-*myo*-inositol **(163)**, in 90% yield. The triol was phosphitylated with bis(2-cyanoethoxy)diisopropylaminophosphine and 1*H*-tetrazole to provide the trisphosphite, which was oxidised using *t*-butylhydroperoxide to give the trisphosphate **(193)** in 80% yield. The allyl ether was cleaved using sodium periodate and ruthenium trichloride hydrate to give the 3-*O*-methylenecarboxylate **(194)**. The hydroxyl and phosphate protective groups were removed by treatment with sodium in liquid ammonia followed by ion exchange chromatography on Q-Sepharose, eluting with a gradient of (TEAB) to provide the pure product in 68% yield.

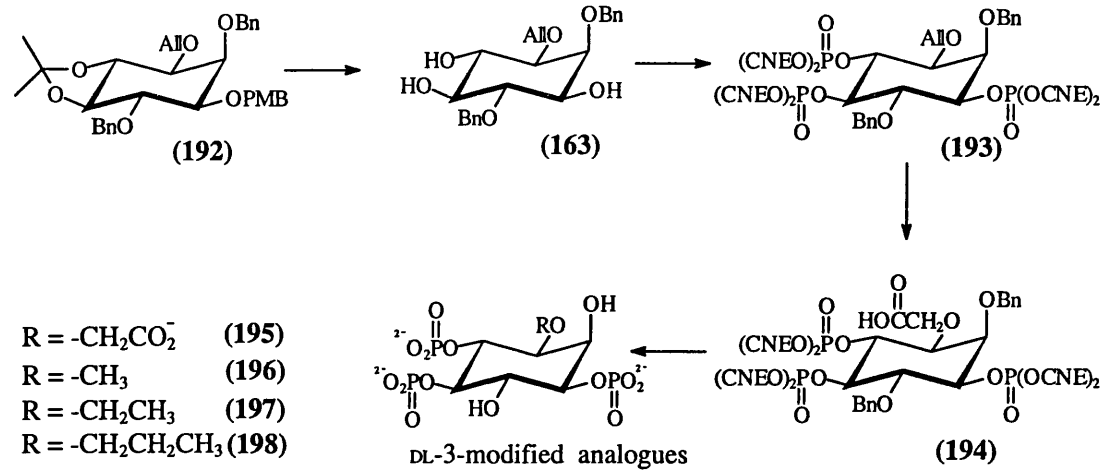


**Figure 54**

DL-Ins(1,4,5)$P_3$-3-*O*-methylenecarboxylate **(195)**, was able to mobilise $Ca^{2+}$ from permeabilised neuroblastoma cells with a potency similar to Ins(1,3,4,5)$P_4$ [$EC_{50}$ value of 3.5μM for **(195)** and $EC_{50}$ value of 2.5μM for Ins(1,3,4,5)$P_4$]. It had a $K_i$ value of 153μM for Ins(1,4,5)$P_3$ 3-kinase from CRBHS and was a potent inhibitor of 5-phosphatase from HEG with a $K_i$ value of 1.0μM.

DL-3-*O*-Methyl-Ins(1,4,5)$P_3$ **(196)**, DL-3-*O*-ethyl-Ins(1,4,5)$P_3$ **(197)** and DL-3-*O*-*n*-propyl-Ins(1,4,5)$P_3$ **(198)** analogues were again prepared from the intermediate DL-3-*O*-allyl-2,6-di-*O*-benzyl-4,5-*O*-isopropylidene-1-*O*-*p*-methoxybenzyl-*myo*-inositol **(192)**. However, in order to synthesise 3-*O*-alkyl derivatives, the allyl function was removed by isomerising to the prop-1-enyl ether using Wilkinsons catalyst, $[(Ph_3)P]_3RhCl$ in the presence of DABCO, in 93% yield. The prop-1-enyl ether was removed using mercuric chloride and mercuric oxide in an acetone/water mixture. The alkyl groups were introduced at the 3-position using methyl, ethyl and *n*-propyl iodide, with sodium hydride as base in DMF. The 4,5-*O*-isopropylidene group and the 1-*O*-*p*-methoxybenzyl ether were removed by refluxing in 1M HCl to produce the corresponding triols. Each of the triols were phosphitylated in the same way as for the synthesis of Ins(1,4,5)$P_3$-3-*O*-methylenecarboxylate and purified by ion exchange chromatography on Q-Sepharose, eluting with a gradient of TEAB and quantified by the Briggs phosphate assay. [430]

DL-3-*O*-Methyl Ins(1,4,5)$P_3$ has an $EC_{50}$ value of 4μM whereas DL-3-*O*-ethyl and DL-3-*O*-*n*-propyl derivatives have an $EC_{50}$ value greater than 100μM. Therefore any hydrophobic group greater than a methyl group has a catastrophic effect on $Ca^{2+}$-release. All three alkylated analogues were potent HEG 5-phosphatase inhibitors with DL-3-*O*-methyl Ins(1,4,5)$P_3$ having a $K_i$ value of 3.3μM and a $K_i$ value for CRBHS 3-kinase inhibition of 192μM. The DL-3-*O*-ethyl and DL-3-*O*-*n*-propyl derivatives were potent HEG 5-phosphatase inhibitors, with $K_i$ values of 11.4μM and 8.0μM respectively.

### 4.4.5 D-Ins(1,4,6)$P_3$ and D-Ins(1,3,6)$P_3$

*myo*-Inositol tetrakisphosphate, Ins(1,3,4,6)$P_4$ **(61)** has been discovered in nature and has also been shown to release $Ca^{2+}$ ions, albeit weakly. [217] Ins(1,3,4,6)$P_4$ has also been shown to be a partial agonist at the Ins(1,4,5)$P_3$ receptor in SH-SY5Y neuroblastoma cells [216] and more recently in rat brain microsomes. [431] [The action of Ins(1,3,4,6)$P_4$ as a partial agonist will be discussed in section 4.9.1].

In order to discover further information concerning the essential features of the activity of Ins(1,3,4,6)$P_4$, two *myo*-inositol trisphosphates, D-Ins(1,4,6)$P_3$ **(199)** and D-Ins(1,3,6)$P_3$ **(200)** [L-Ins(1,3,4)$P_3$] can be derived by removing the phosphate at the D-3 and D-4 positions and provide the same ring pucker conformation as D-Ins(1,4,5)$P_3$ with different orientations of the hydroxyl moiety. Similarly L-Ins(1,4,6)$P_3$ **(201)** and L-Ins(1,3,6)$P_3$ **(202)** may be considered as having the same ring pucker conformation as L-Ins(1,4,5)$P_3$ but with different hydroxyl orientations.

The synthesis of D-Ins(1,4,6)$P_3$ by Ozaki and coworkers has briefly been discussed in section 3.7.1. They found that D-Ins(1,4,6)$P_3$ had a rotation $[\alpha]_D = -8.9°$ (c = 0.90, $H_2O$), as the sodium salt. [432] However, the pH was not stated. Hirata and coworkers [433] found that D-Ins(1,4,6)$P_3$ bound to the Ins(1,4,5)$P_3$ receptor of rat basophilic leukemic cells (RBL) some 16-fold less potently than Ins(1,4,5)$P_3$, but was 125-fold less potent at releasing $Ca^{2+}$ as Ins(1,4,5)$P_3$. The potency of D-Ins(1,4,6)$P_3$ with respect to binding was found to be the same as for Ins(1,3,4,6)$P_4$. Thus the phosphate groups at the 1-, 4- and 6-positions in the D-configuration was essential for activity. D-Ins(1,4,6)$P_3$ was assayed for 5-phosphatase activity from erythrocyte ghosts and inhibited the enzyme with a $K_i$ value of 9.2μM. D-Ins(1,4,6)$P_3$ was also as potent as

$Ins(1,4,5)P_3$ in inhibiting the phosphorylation of $[^3H]Ins(1,4,5)P_3$ but although no clear values for this inhibition were tabulated, it appeared to be less than 5μM.

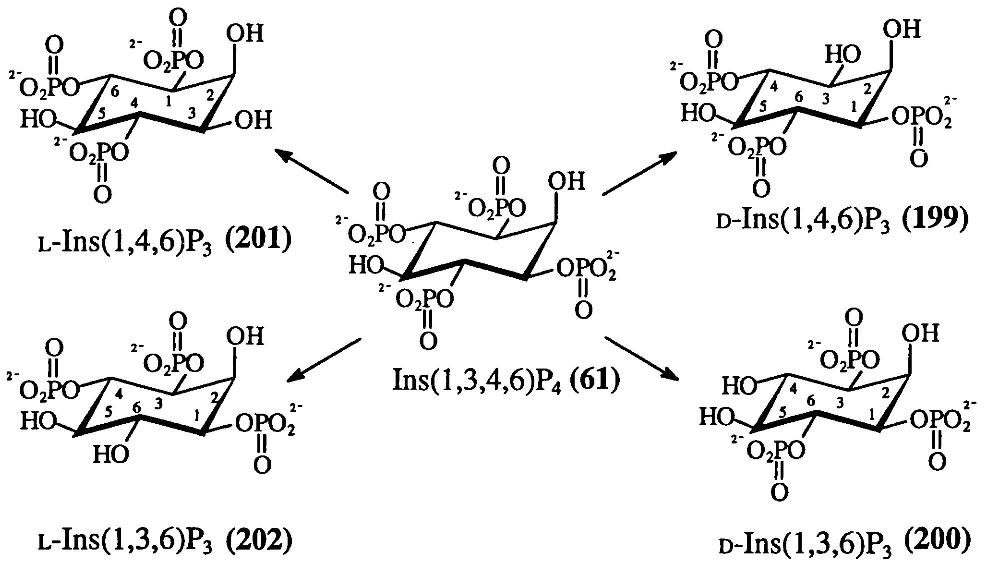


**Figure 55**

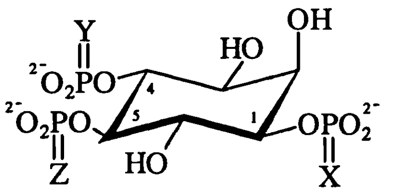
D-$Ins(1,3,4)P_3$ **(202)** was reported by Ozaki and coworkers to have a specific rotation $[\alpha]_D$ of -6° (isolated as the hexa-ammonium salt, but no biological data was given at that time. Later, Gou and Chen [434] and Chen and coworkers [376] reported a chemoenzymatic synthesis of D-$Ins(1,3,4)P_3$ (as the hexa-potassium salt) which had a $[\alpha]_D$ of +14° (c = 2, $H_2O$, pH 8.2). This caused confusion, so Potter and coworkers [435] unambiguously synthesised both enantiomers of $Ins(1,3,4)P_3$. It was found that D-$Ins(1,3,4)P_3$ had a $[\alpha]_D$ of +37° (c= 2, 1M TEAB, pH 7.8) and L-$Ins(1,3,4)P_3$ had a $[\alpha]_D$ of -40° (c = 0.42, 1M TEAB, pH 7.8) both calculated for the free acid. It was found that Gou and coworkers were correct in the assignment of the optical rotation, which may cast doubt on the work of Ozaki and coworkers.

Hirata and coworkers [433] have reported some biological data for the synthetic L-$Ins(1,3,4)P_3$ (which was synthesised according to Ozaki and coworkers [350]). It was found that L-$Ins(1,3,4)P_3$ could bind to the $Ins(1,4,5)P_3$ receptor from rat basophilic leukemic (RBL) cells some 90-fold weaker than $Ins(1,4,5)P_3$, but was 3000-fold weaker at $Ca^{2+}$-release and the $EC_{50}$ value could not be determined. The poor binding and $Ca^{2+}$-release experiments may call into question the true identity of Ozaki's L-$Ins(1,3,4)P_3$, unless L-$Ins(1,3,4)P_3$ is a partial agonist in this particular cell type? L-$Ins(1,3,4)P_3$ was found to have a $K_i$ value of 27.4μM and was less potent than D-$Ins(1,4,6)P_3$ at inhibiting 3-kinase. However, in contrast, the two enantiomers of $Ins(1,3,4)P_3$ (Figure 55) (synthesised according to Potter and coworkers) [435] were examined in *Limulus* ventral photoreceptors. [435] It was shown that D-$Ins(1,3,4)P_3$

was much less efficient for $Ca^{2+}$-release than D-Ins(1,4,5)$P_3$ or L-Ins(1,3,4)$P_3$. In addition, and unlike D-Ins(1,4,5)$P_3$, L-Ins(1,3,4)$P_3$ showed repetitive bursts of depolarisation which continued for up to 5min, suggesting that L-Ins(1,3,4)$P_3$ was metabolised more slowly than D-Ins(1,4,5)$P_3$. In conclusion, the biological actions of D-Ins(1,3,4)$P_3$ should be re-examined.

### 4.5 *myo*-Inositol Phosphorothioates, Chemistry and Pharmacology

The same protected precursors that have been used for the synthesis of *myo*-inositol phosphates can also be used to synthesise *myo*-inositol phosphorothioates. The difference being the P(III) phosphite is oxidised with sulphur in pyridine [391] or phenacetyl disulphide, [392] to give the fully protected phosphorothioate triester. Deprotection of the fully protected phosphorothioate takes place by chemical reduction using sodium in liquid ammonia followed by further purification by ion exchange chromatography to afford the pure deprotected compound.



X = Y = Z = S (203)
X = Y = O; Z = S (204)
X = O; Y = Z = S (205)
X = S; Y = Z = O (206)

**Figure 56**

The first reported Ins(1,4,5)$P_3$ analogue was racemic *myo*-inositol 1,4,5-trisphosphorothioate Ins(1,4,5)$PS_3$ **(203)**. This was synthesised in the same way as racemic Ins(1,4,5)$P_3$ [391] except that oxidation of the trisphosphite was effected with sulphur in pyridine instead of *t*-butylhydroperoxide. The deprotection of the phosphorothioate was then carried out in one step using sodium in liquid ammonia, and purified by ion exchange chromatography to give Ins(1,4,5)$PS_3$. Several other *myo*-inositol phosphorothioate analogues have been synthesised and evaluated, (Figure 56). These include the following: DL-*myo*-inositol 1,4-bisphosphate-5-phosphorothioate DL-Ins(1,4,5)$P_3$-5S **(204)**, DL-*myo*-inositol 1-phosphate-4,5-bisphosphorothioate DL-Ins(1,4,5)$P_3$-4,5$S_2$ **(205)** and DL-*myo*-inositol 4,5-bisphosphate-1-phosphorothioate DL-Ins(1,4,5)$P_3$-1S **(206)** all of which are based on Ins(1,4,5)$P_3$. After the synthesis and biological evaluation of DL-Ins(1,4,5)$PS_3$ a closer analogue, DL-Ins(1,4,5)$P_3$-5S was synthesised and evaluated. [390,436] It was found that substitution with a phosphorothioate only at the 5-position slightly decreased the effective $Ca^{2+}$-mobilising properties with an $EC_{50}$ value of 0.8μM compared to 0.11μ M for Ins(1,4,5)$P_3$, [437] in permeabilised SH-SY5Y human neuroblastoma cells. DL-

$Ins(1,4,5)P_3$-5S was also a potent inhibitor of 5-phosphatase from HEG with a $K_i$ value of 6.8μM, but appeared to be a substrate for the 3-kinase enzyme from CRBHS and inhibited the phosphorylation of $[^3H]Ins(1,4,5)P_3$ with a $K_i$ value of 5μM. DL-$Ins(1,4,5)P_3$-4,5$S_2$ was prepared in a similar way to DL-$Ins(1,4,5)P_3$-5S, using DL-1,2,4-tri-*O*-benzyl-3-*O*-[di(2,2,2-trichloroethoxyphospho)]-*myo*-inositol as the starting material. [438] This intermediate was phosphitylated at the free hydroxyl 4- and 5-positions followed by oxidation with sulphur in pyridine, deblocked and purified in the same way as DL-$Ins(1,4,5)P_3$-5S in order to give DL-$Ins(1,4,5)P_3$-4,5$S_2$. This analogue mobilised $Ca^{2+}$ from SH-SY5Y neuroblastoma cells with an $EC_{50}$ value of 1.2μM slightly higher than DL-$Ins(1,4,5)P_3$-5S, but lower than DL-$Ins(1,4,5)PS_3$ ($EC_{50}$ value of 2.5μM) indicating that sequential phosphorothioate replacement decreases the $Ca^{2+}$-mobilising ability, albeit slightly. The analogue was a potent inhibitor of 5-phosphatase from HEG with a $K_i$ value of 1.4μM and was found to be a cosubstrate for 3-kinase from CRBHS inhibiting the metabolism of $Ins(1,4,5)P_3$ with a $K_i$ value of 46μM. DL-$Ins(1,4,5)P_3$-4,5$S_2$ has also been used to prepare the analogue DL-*myo*-inositol 1-phosphate-4,5-pyrophosphate DL-$Ins(1,4,5)P_3$-(PP) which provided further evidence for the free rotation of the crucial 4,5-bisphosphate motif. This analogue showed no $Ca^{2+}$-releasing activity and little inhibition of 3-kinase and 5-phosphatase: (3-kinase, $K_i$ value of >100μM and 5-phosphatase $K_i$ value of 200μM). [422]

Increasing the number of phosphorothioate groups in the molecule tended to increase the $EC_{50}$ value as well as increasing its resistance to 5-phosphatase from HEG. However, DL-$Ins(1,4,5)P_3$-4,5$S_2$ was slightly more potent than DL-$Ins(1,4,5)PS_3$ ($K_i$ value of 1.7μM) and was a poor inhibitor of 3-kinase from CRBHS with a $K_i$ value of 230μM. This indicated the specificity of a phosphate moiety over a phosphorothioate group for 3-kinase interaction. Another analogue, DL-*myo*-inositol 4,5-bisphosphate-1-phosphorothioate DL-$Ins(1,4,5)P_3$-1S, has also been synthesised in racemic [439] and optically active [358] form. The phosphorothioate group at this position offers a point of attachment for reporter groups such as fluorescent and spin labels, due to the nucleophilic nature of the sulphur atom. DL-$Ins(1,4,5)P_3$-1S was modified by coupling with the fluorescent reporter group nitrobenzoxadiazole (NBD) moiety at the 1-position to provide DL-NBD-$Ins(1,4,5)P_3$. It was found that the analogue could bind to the $Ins(1,4,5)P_3$ cerebellar receptor with high affinity and it released $Ca^{2+}$ potently with an $EC_{50}$ value only 6-fold higher than for $Ins(1,4,5)P_3$. Other research groups have modified the 1-position and 2-hydroxyl by attaching probes or affinity matrices, but this will not be discussed any further. This research has been reviewed in two references. [408,409]

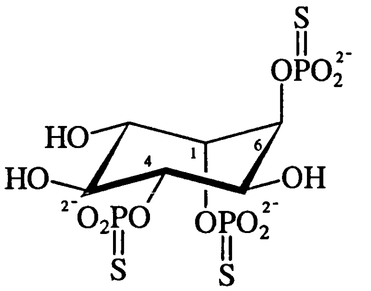
### 4.5.1 Biology and Application of $Ins(1,4,5)PS_3$

DL-$Ins(1,4,5)PS_3$ is recognised by $Ins(1,4,5)P_3$ receptors from many cell-types and is a full agonist for $Ca^{2+}$ release and is resistant to all known $Ins(1,4,5)P_3$ metabolic pathways. The function of $Ins(1,4,5)P_3$ in the red blood cell is unknown at present even though phosphoinositide breakdown is well established. It is known that in permeabilised red blood cells, $Ins(1,4,5)P_3$ elicits the transient release of $Ca^{2+}$, disorganisation of the spectrin network and reversible shape changes, which were measured by immunofluorescence. [440] However, DL-$Ins(1,4,5)PS_3$, being resistant to metabolic degradation, gave a sustained $Ca^{2+}$ release and irreversible shape changes and disorganisation of the spectrin network. Thus the $Ins(1,4,5)P_3$ pathway plays a pivotal role in the shape maintenance of red blood cells.

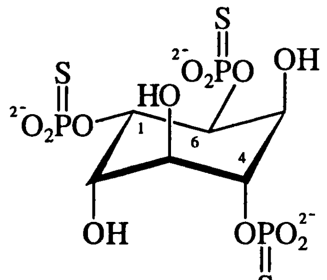
A further application of DL-$Ins(1,4,5)PS_3$ has been to demonstrate the 'quantal' release of $Ca^{2+}$ by $Ins(1,4,5)P_3$ in permeabilised hepatocytes. [441] It was demonstrated that the amount of $Ca^{2+}$ released from the $Ins(1,4,5)P_3$-sensitive $Ca^{2+}$ store iwas dependent on the concentration of $Ins(1,4,5)P_3$. Submaximal concentrations of $Ins(1,4,5)P_3$ or DL-$Ins(1,4,5)PS_3$ failed to empty $Ca^{2+}$ stores completely and this was not due to deactivation of the stimulus nor receptor desensitisation because of the enzymatic stability of DL-$Ins(1,4,5)PS_3$.

The horseshoe crab *Limulus*, possesses microvillar photoreceptors which release $Ca^{2+}$ on exposure to light, *via* the $Ins(1,4,5)P_3$ pathway. DL-$Ins(1,4,5)PS_3$ has been used to investigate the mechanisms that terminate $Ca^{2+}$ mobilisation in ventral photoreceptors of *Limulus*. DL-$Ins(1,4,5)PS_3$ generated unusual sustained repetitive oscillations of $Ca^{2+}$-dependent membrane potential which may persist for tens of minutes. [442] Finally, in rat pancreatic acinar cells, DL-$Ins(1,4,5)PS_3$ has been used to distinguish between $Ins(1,4,5)P_3$-sensitive and $Ins(1,4,5)P_3$-insensitive non-mitochondrial MgATP-dependent $Ca^{2+}$ pools. [443] The analogue was used to keep the $Ins(1,4,5)P_3$-sensitive $Ca^{2+}$ store empty and so no $Ca^{2+}$-reuptake occurred into the $Ins(1,4,5)P_3$-insensitive pool. However, in permeabilised rat parotid acinar cells, there is evidence to show $Ca^{2+}$-reuptake into $Ins(1,4,5)P_3$-sensitive and thapsigargin-sensitive $Ca^{2+}$ store in the presence of DL-$Ins(1,4,5)PS_3$. [444]

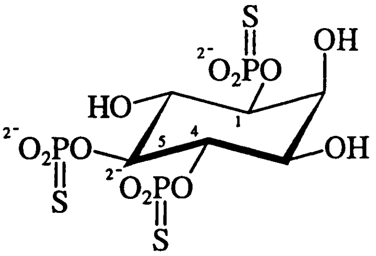
### 4.5.2 L-*chiro*-Ins(1,4,6)$PS_3$, L-Ins(1,4,5)$PS_3$ and Ins(1,3,5)$PS_3$



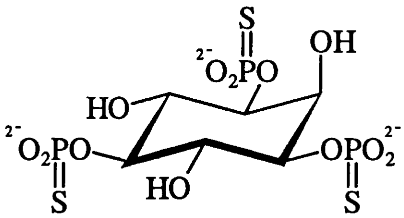
L-*chiro*-Ins(1,4,6)$PS_3$ (**207**)



alternative conformation (**208**)



L-Ins(1,4,5)$PS_3$ (**209**)



Ins(1,3,5)$PS_3$ (**210**)

**Figure 57**

The analogue L-*chiro*-inositol 1,4,6-trisphosphorothioate (**207**), L-*chiro*-Ins(1,4,6)$PS_3$, was synthesised in the same way as L-*chiro*-Ins(1,4,6)$P_3$ except that sulphoxidation occurred (using sulphur in pyridine) to give the totally protected phosphorothioate triester, in 90% yield. [424] Deprotection using sodium in liquid ammonia and purification by ion-exchange chromatography on Q-Sepharose Fast Flow, gave L-*chiro*-Ins(1,4,6)$PS_3$ in 70% yield. The analogue did not interact with the Ins(1,4,5)$P_3$ receptor from electrically permeabilised SH-SY5Y human neuroblastoma cells and was poorly recognised by the 3-kinase from CRBHS with a $K_i$ value >100μM. However, L-*chiro*-Ins(1,4,6)$PS_3$ which has little similarity to Ins(1,4,5)$P_3$ inhibited 5-phosphatase from HEG with a $K_i$ value of 0.3μM which makes it a potent and selective 5-phosphatase inhibitor. An explanation for this inhibition has been proposed [422] which considered the *chiro*-inositol ring to flip so that the 1- and 6-phosphorothioate groups were equatorial but the 4-phosphorothioate, and the 2-, 3- and 5-hydroxyl groups axial (**208** in Figure 57). Molecular modelling has shown good overlay between the phosphorothioates of L-*chiro*-Ins(1,4,6)$PS_3$ and the phosphates of Ins(1,4,5)$P_3$, the major difference being the pseudo-axial 1-phosphorothioate compared to Ins(1,4,5)$P_3$. In another important study, [425] L-*chiro*-Ins(1,4,6)$PS_3$ competitively inhibited the dephosphorylation of Ins(1,4,5)$P_3$, but surprisingly, was found to inhibit the cleavage of the 5-phosphate group from Ins(1,3,4,5)$P_4$

noncompetitively with apparent $K_i$ values of 0.67 and 0.43μM respectively. This analogue was the first phosphorothioate of Ins(1,4,5)$P_3$ to show noncompetitive kinetics for inhibition of Ins(1,4,5)$P_3$ metabolism. These experiments suggest that Ins(1,3,4,5)$P_4$ and L-*chiro*-Ins(1,4,6)$PS_3$ may bind to different regions on the 5-phosphatase from porcine skeletal muscle. Although the noncompetitive inhibition may indicate enzyme inactivation by L-*chiro*-Ins(1,4,6)$PS_3$, or the presence of soluble type I 5-phosphatase in muscle.

L-*myo*-Inositol 1,4,5-trisphosphorothioate, L-Ins(1,4,5)$PS_3$ **(209)** was synthesised from D-1,2,4-tri-*O*-benzyl-*myo*-inositol which in turn was obtained from the L-enantiomer of 1-*O*-allyl-2,3,6-tri-*O*-benzyl-*myo*-inositol. [424] Thus, the allyl group was first isomerised to give the *cis*-prop-1-enyl ether using potassium *t*-butoxide in dry DMSO followed by acid hydrolysis to provide D-1,2,4-tri-*O*-benzyl-*myo*-inositol. The triol was phosphitylated with bis(2-cyanoethoxy)diisopropylaminophosphine and 1*H*-tetrazole followed by sulphoxidation using sulphur in pyridine to give the fully protected trisphosphorothioate triester in 99% yield. Deprotection and purification as for other phosphorothioates gave L-Ins(1,4,5)$PS_3$ in 91% yield. This analogue did not interact with the Ins(1,4,5)$P_3$ receptor from electrically permeabilised SH-SY5Y human neuroblastoma cells and was poorly recognised by 3-kinase from CRBHS with a $K_i$ value of 108μM. However, L-Ins(1,4,5)$PS_3$ was a potent inhibitor of 5-phosphatase from HEG preparation with a $K_i$ value of 0.43μM. Thus, there was a 250-fold enantiomeric selectivity over 3-kinase inhibition and a 35-fold increase in affinity of over L-Ins(1,4,5)$P_3$ for 5-phosphatase inhibition.

The *meso* compound *myo*-inositol 1,3,5-trisphosphorothioate, Ins(1,3,5)$PS_3$ **(210)**, was synthesised using the symmetry of the orthoformate structure. [424] The hydroxyl groups at the 2-, 4- and 6-positions were *p*-methoxybenzylated with *p*-methoxybenzyl chloride in DMF and sodium hydride as base to give 2,4,6-tri-*O*-*p*-methoxybenzyl-*myo*-inositol orthoformate in 78% yield. The 1-, 3- and 5-positions were exposed by refluxing the protected intermediate in a mixture of 2M HCl and methanol (1:20) to give 2,4,6-tri-*O*-*p*-methoxybenzyl-*myo*-inositol in 85% yield. The 1,3,5-triol was phosphitylated with bis(benzyloxy)diisopropylaminophosphine and 1*H*-tetrazole in dichloromethane followed by sulphoxidation with sulphur in pyridine to give the totally protected 1,3,5-trisphosphorothioate. Deprotection and purification over Q-Sepharose gave pure Ins(1,3,5)$PS_3$ in 91% yield.

Ins(1,3,5)$PS_3$ **(210)**, did not interact with the Ins(1,4,5)$P_3$ receptor from electrically permeabilised SH-SY5Y human neuroblastoma cells. This analogue was poorly recognised by the 3-kinase from CRBHS with a $K_i$ value of 247μM, but was efficiently recognised by 5-phosphatase from (HEG) preparation with a $K_i$ value of 0.52μM. This showed a 475-fold specificity of 5-phosphatase over 3-kinase for Ins(1,3,5)$PS_3$.

### 4.6 Fluoro- and other Halogenated Ins(1,4,5)$P_3$ Analogues

There have been several reports of racemic and chiral fluorinated *myo*-inositol phosphate analogues, together with other 3-modified halogen (Cl, Br) analogues.

#### 4.6.1 3-X-modified Ins(1,4,5)$P_3$ Analogues (X = F, Cl, Br)

The main compound of interest has been D-3-deoxy-3-fluoro-*myo*-inositol 1,4,5-trisphosphate (**217** in Figure 58) D-3F-Ins(1,4,5)$P_3$ which was prepared from D-3-deoxy-3-fluoro-*myo*-inositol (**211**). This intermediate was prepared from the chiral starting material L-quebrachitol. When L-quebrachitol was treated with neat diethylaminosulphur trifluoride (DAST) at 20°C a mixture of D-3-deoxy-3-fluoro-1-*O*-methyl-*myo*-inositol and D-3-deoxy-3-fluoro-4-*O*-methyl-*myo*-inositol was produced. The methyl group of both compounds was removed using boron tribromide in dichloromethane to provide D-3-deoxy-3-fluoro-*myo*-inositol (**211**) in 35-50% overall yield. [445]

When D-3-deoxy-3-fluoro-*myo*-inositol was treated with 2-methoxypropene and camphorsulphonic acid in DMF at 80°C for 40h a mixture of 1,2:4,5-(**212**) and 1,2:5,6-di-*O*-isopropylidene acetals were produced (83% yield) in a ratio of 2:3 respectively. Only 3-fluoro-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol (**212**) was used in the synthesis and the other regioisomer was recycled. The free 6-hydroxyl group was benzylated to give (**213**) and the *trans*-acetal was selectively cleaved using HCl in methanol in order to expose the 4- and 5-hydroxyl groups which were then benzoylated using benzoyl chloride and DMAP in pyridine to provide derivative (**214**) in (92% yield). The *cis*-1,2-*O*-isopropylidene acetal was removed with acetic acid/water/THF (2:1:1), 80°C for 14h in 92% yield, followed by selective benzoylation at the 1-position using benzoyl chloride/pyridine at 0°C for 12h in 92% yield. The exposed 2-hydroxyl group was protected as the ethoxyethyl derivative in 86% yield which gave the fully protected compound (**215**). Basic hydrolysis of the three benzoate ester groups at D-1-, 4- and 5-positions using methanolic potassium carbonate 23°C for 14h exposed the hydroxyl groups at these positions in 83% yield. Phosphorylation was accomplished using tetrabenzylpyrophosphate and sodium hydride in DMF at 0°C for 8-10h to give the totally protected phosphorylated intermediate **(216)** in 95% yield. The ethoxyethyl moiety was cleaved using toluene-*p*-sulphonic acid in methanol (23°C for 14h) in 82% yield. Debenzylation was accomplished by hydrogenolysis over platinum oxide and titration with NaOH gave the stable hexasodium salt of D-3F-Ins(1,4,5)$P_3$. [446] The analogues D-3-chloro-*myo*-inositol 1,4,5-trisphosphate, [D-3Cl-Ins(1,4,5)$P_3$] and D-3-bromo-*myo*-inositol 1,4,5-trisphosphate, [D-3Br-Ins(1,4,5)$P_3$] were prepared by a similar route from their respective D-3-halogen-*myo*-inositol derivatives.

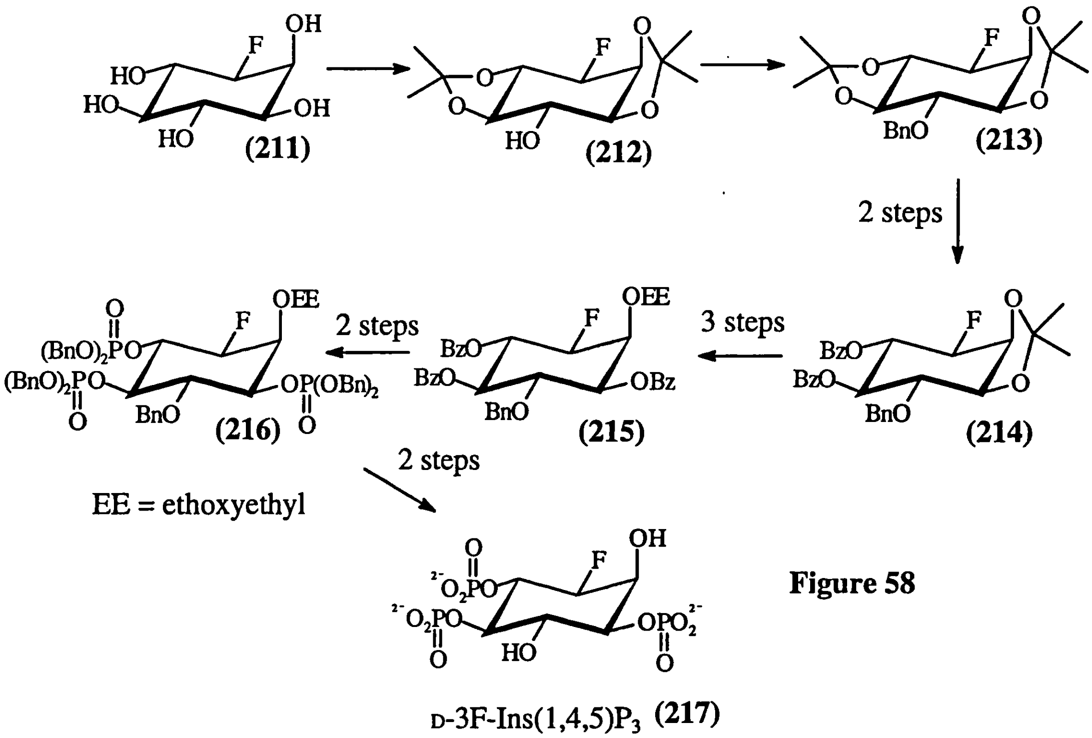



**Figure 58**

The $EC_{50}$ value for Ins(1,4,5)$P_3$ was found to be 52.1nM for the release of $Ca^{2+}$ from SH-SY5Y human neuroblastoma cells. D-3F-Ins(1,4,5)$P_3$ was found to have an $EC_{50}$ value of 120.2nM, D-3Cl-Ins(1,4,5)$P_3$ an $EC_{50}$ value of 639.8nM and D-3Br-Ins(1,4,5)$P_3$ an $EC_{50}$ value of 1100nM. For the 3-modified halogen derivatives of Ins(1,4,5)$P_3$, one may suggest that the Ins(1,4,5)$P_3$ receptor is sensitive to steric bulk, similar to the 3-*O*-alkylated Ins(1,4,5)$P_3$ molecules (discussed in section 4.4.4). A fluorine substituent has a volume of 10.1$Å^3$, chlorine 19.4$Å^3$ and bromine has a volume of 25.2$Å^3$. Moreover, the increase in bulk volume at the 3-position, switches from being Ins(1,4,5)$P_3$-like [seen for D-3F-Ins(1,4,5)$P_3$] to being Ins(1,3,4,5)$P_4$-like [seen for D-3Br-Ins(1,4,5)$P_3$] with D-3Cl-Ins(1,4,5)$P_3$ being intermediate in size, between Ins(1,4,5)$P_3$ and Ins(1,3,4,5)$P_4$. It may be concluded that the phosphorylation of Ins(1,4,5)$P_3$ $\rightarrow$ Ins(1,3,4,5)$P_4$ may act to dissect the function of these two molecules at the Ins(1,4,5)$P_3$ receptor. [447]

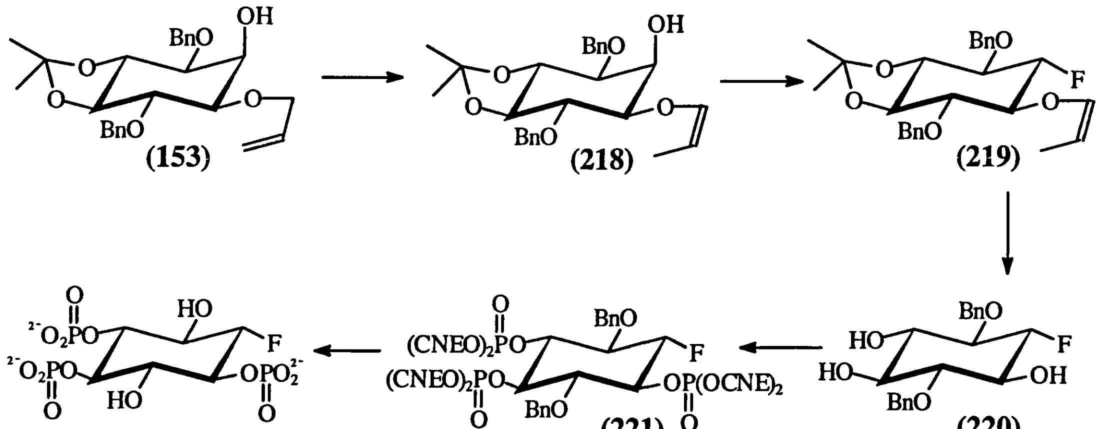
The analogue D-3F-Ins(1,4,5)$P_3$ has also been tested on the enzymes 5-phosphatase and 3-kinase. It appeared to be resistant to 3-kinase from CRBHS with a $K_i$ value of 8.6μM. However, D-3F-Ins(1,4,5)$P_3$ was hydrolysed by 5-phosphatase from HEG at a similar rate to that for Ins(1,4,5)$P_3$ but inhibited the dephosphorylation of [$^3$H]Ins(1,4,5)$P_3$ with a $K_i$ value of 3.9μM. [421]

D-3F-Ins(1,4,5)$P_3$ has recently been used as a tool in order to investigate $Ca^{2+}$-influx in rat hepatocytes. Microinjection of Ins(1,3,4,5)$P_4$, Ins(1,4,5)$P_3$ or D-3F-Ins(1,4,5)$P_3$ indicated that Ins(1,4,5)$P_3$ rather than Ins(1,3,4,5)$P_4$ is responsible for $Ca^{2+}$-influx. When the agonist vasopressin activated PLC, an influx of $Mn^{2+}$ was observed which was independent of intracellular $Ca^{2+}$ stores, if the depletion was delayed by using low agonist concentration or heparin. It was proposed [448] that Ins(1,4,5)$P_3$ was able to stimulate $Ca^{2+}$-influx by two pathways. Thus Ins(1,4,5)$P_3$ activates receptor-operated $Ca^{2+}$-channels directly. The $Ca^{2+}$-influx resulting from this is followed by Ins(1,4,5)$P_3$-induced depletion of $Ca^{2+}$ stores, producing a 'store-dependent-entry'.

### 4.6.2 2-Modified Fluoro-Inositol Phosphates

The intermediate DL-1-*O*-allyl-3,6-di-*O*-benzyl-4,5-*O*-isopropylidene-*myo*-inositol **(153)** was prepared from *myo*-inositol in several transformations according to Gigg and coworkers. [383] This intermediate was used to synthesise all the 2-fluorinated inositol trisphosphate analogues in this section. 2-Deoxy-2-fluoro-*scyllo*-inositol 1,4,5-trisphosphate (**222** in Figure 59) was synthesised in 5 steps from this intermediate. First, the allyl group was isomerised using potassium *t*-butoxide in dry DMSO for 3h to give the *cis*-prop-1-enyl ether **(218)** in quantitative yield. The equatorial fluorine group was introduced using DAST (3 equivalents) in dry dichloromethane *via* an $S_N2$ reaction to give DL-3,6-di-*O*-benzyl-2-deoxy-2-fluoro-4,5-*O*-isopropylidene-*scyllo*-inositol **(219)** in 68% yield. [385] The acid sensitive 4,5-*O*-isopropylidene group and the *cis*-prop-1-enyl were removed under acidic conditions [1M HCl-methanol, (1:5)], to give the racemic 1,4,5-triol **(220)** in 89% yield. Phosphitylation with bis(2-cyanoethoxy)diisopropylaminophosphine and 1*H*-tetrazole followed by oxidation with *t*-butylhydroperoxide provided the trisphosphate triester **(221)** which was not isolated. The protective groups were removed using sodium in liquid ammonia, and purified by ion exchange chromatography to give racemic 2-F-*scyllo*-Ins(1,4,5)$P_3$ **(222)** in 34% yield. It was found to be a full agonist for the release of $Ca^{2+}$ from permeabilised SH-SY5Y human neuroblastoma cells with an $EC_{50}$ value of 0.77μM. The analogue was found to be a substrate albeit a poor one for 5-

phosphatase from HEG with a $K_i$ value of 0.7μM, but was resistant to 3-kinase from CRBHS with a $K_i$ value of 8.8μM. [449]




2F-*scyllo*-Ins(1,4,5)$P_3$ **(222)**

**Figure 59**

DL-2-Deoxy-2,2-difluoro-*myo*-inositol 1,4,5-trisphosphate **(227)**, DL-2,2$F_2$-*scyllo*-Ins(1,4,5)$P_3$ was synthesised from the same intermediate **(153)**, and subsequently resolved to give the D-**(228)** and L-**(229)** enantiomers (in Figure 60). [385] The 2-hydroxyl group was oxidised using DMSO/acetic anhydride in 94% yield in order to provide the 2-inosose derivative **(223)**. The 2-deoxy-2,2-difluoro moiety was introduced using excess DAST (4 equivalents) in dry dichloromethane (79% yield) to give compound **(224)**. The allyl group was isomerised to a mixture of *cis*- and *trans*-prop-1-enyl ethers (9:2) using Wilkinson's catalyst to give **(225)** in 75% yield and the acid sensitive protective groups were removed under acidic conditions to provide the triol **(226)**. The exposed 1-, 4- and 5-hydroxyl groups were phosphorylated and deprotected in the same way as for 2F-*scyllo*-Ins(1,4,5)$P_3$ to give DL-2,2$F_2$-Ins(1,4,5)$P_3$.

Chiral D-**(228)** and L-2,2$F_2$-Ins(1,4,5)$P_3$ **(229)** were synthesised by resolving DL-3,6-di-*O*-benzyl-2-deoxy-2,2-difluoro-4,5-*O*-isopropylidene-*myo*-inositol **(74)**, using 1*S*-(-)-ω-camphanic acid chloride. [385] The diastereoisomeric camphanate ester derivatives were separated by flash chromatography (described in section 3.7). The camphanate was then hydrolysed under basic conditions in >90% yield for both compounds, and the 4,5-*O*-isopropylidene group was removed under acidic conditions followed by phosphorylation/deprotection as for 2F-*scyllo*-Ins(1,4,5)$P_3$ to give the resolved compounds D-**(228)** and L-2,2$F_2$-Ins(1,4,5)$P_3$ **(229)**.

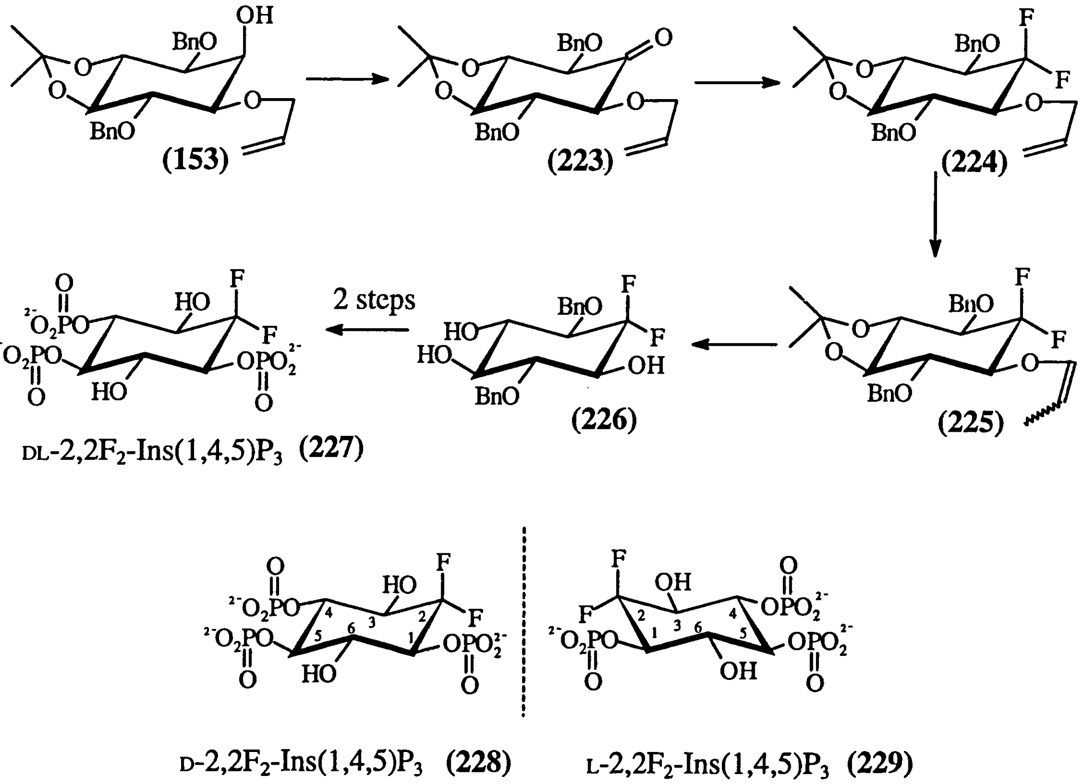



**Figure 60**

The $EC_{50}$ value for DL-2,2$F_2$-Ins(1,4,5)$P_3$ was found to be 0.41μM, for the D-enantiomer 0.21μM and for the L-enantiomer 53μM. Thus, the D-enantiomer is a full agonist for $Ca^{2+}$-release from SH-SY5Y human neuroblastoma cells and is 250-fold more potent than the L-enantiomer, indicating the stereospecificity for the D-enantiomer at the Ins(1,4,5)$P_3$ receptor. [449] The three compounds were then tested for 5-phosphatase HEG preparation and 3-kinase CRBHS. DL-2,2$F_2$-Ins(1,4,5)$P_3$ showed inhibition of 5-phosphatase with a $K_i$ value of 26μM [the $K_m$ value for Ins(1,4,5)$P_3$ had a value of 40μM in this study]. D-2,2$F_2$-Ins(1,4,5)$P_3$ was a substrate for 5-phosphatase and inhibited [$^3$H]Ins(1,4,5)$P_3$ dephosphorylation with a $K_i$ value of 60μM. However, L-2,2$F_2$-Ins(1,4,5)$P_3$ was resistant to 5-phosphatase with a $K_i$ value of 19μM.

The 3-kinase enzyme recognised D-2,2$F_2$-Ins(1,4,5)$P_3$ as a substrate with high affinity which inhibited the phosphorylation of [$^3$H]Ins(1,4,5)$P_3$ competitively with a $K_i$ value of 6.6μM. However, L-2,2$F_2$-Ins(1,4,5)$P_3$ was a potent inhibitor of 3-kinase and inhibited the phosphorylation of [$^3$H]Ins(1,4,5)$P_3$ with a $K_i$ value of 11.5μM, which compares favourably with Ins(1,4,5)$P_3$ with a $K_m$ value of 3.2μM. [450]

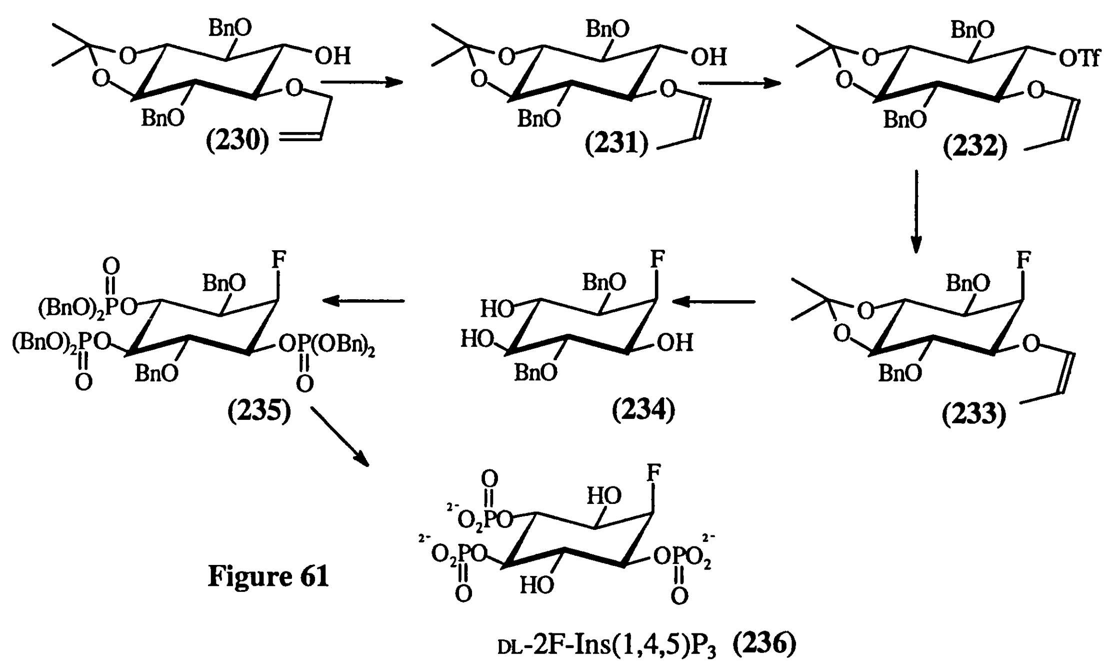


Figure 61

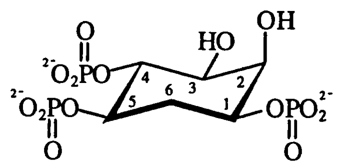
A final 2-modified fluorinated analogue, DL-2-deoxy-2,-fluoro-*myo*-inositol 1,4,5-trisphosphate DL-2F-Ins(1,4,5)$P_3$ (**236** in Figure 61), has been successfully synthesised. [428] The intermediate DL-1-*O*-allyl-3,6-di-*O*-benzyl-4,5-*O*-isopropylidene-*scyllo*-inositol **(230)** was used as the intermediate to synthesise DL-2F-Ins(1,4,5)$P_3$. The allyl group was isomerised using potassium *t*-butoxide in dry DMSO to give the *cis*-prop-1-enyl ether **(231)**. The 2-hydroxyl was then set-up for $S_N2$ *via* the trifluoromethanesulphonate **(232)** followed by fluoride inversion using anhydrous tetrabutylammonium fluoride to restore the *myo*-configuration in 69% yield. The observation that $^3J_{HF}$ was 29.5Hz in the spectrum of DL-1-*O*-*cis*-prop-1-enyl-2-deoxy-2-fluoro-3,6-di-*O*-benzyl-4,5-*O*-isopropylidene-*myo*-inositol **(233)** confirmed the inversion of the 2-position back to the *myo*-configuration. Removal of the acid sensitive protective groups gave the triol **(234)** and phosphitylation [using bis(benzyloxy)diisopropylaminophosphine and 1*H*-tetrazole] followed by oxidation with *t*-butylhydroperoxide, provided the fully protected trisphosphate **(235)**. Deprotection with sodium in liquid ammonia gave DL-2F-Ins(1,4,5)$P_3$ **(236)** in 41% yield. The racemic compound has an $EC_{50}$ value of 104.7nM, [Ins(1,4,5)$P_3$ had a value of 51.6nM] from SH-SY5Y human neuroblastoma cells. DL-2F-Ins(1,4,5)$P_3$ was also resistant to 5-phosphatase inhibition and had a $K_i$ value of 14.4μM, but was susceptible to 3-kinase activity, and inhibited phosphorylation of [$^3$H]Ins(1,4,5)$P_3$ with a $K_i$ value of 3.0μM.

Other fluorinated inositol phosphates have been synthesised including DL-6-deoxy-6-fluoro-*myo*-inositol 1,4,5-trisphosphate by Ley and coworkers [401] together with DL-6-deoxy-6-fluoro-*chiro*-inositol 2,3,5-trisphosphate by Carless and coworkers [451] and

DL-2-deoxy-2,2-difluoro-*myo*-inositol 1,3,4-trisphosphate by Prestwich and coworkers. [452] All three of these compounds have no biological data associated with the synthesis.

## 4.7 D-6-Deoxy-Ins(1,4,5)$P_3$

D-6-Deoxy-*myo*-inositol 1,4,5-trisphosphate D-6-deoxy-Ins(1,4,5)$P_3$ **(237)**, was synthesised from D-galactose (D. Dubreuil, J. Cleophax, B. V. L. Potter and S. D. Gero, *unpublished data*). However, Ley and coworkers have synthesised this analogue in racemic form from benzene. [401] Briefly, the intermediate, epoxy acetal **(238)** which was derived from the versatile *cis*-1,2-diol was treated with lithium aluminium hydride in ether at reflux temperature to give the 6-deoxy compound **(239)** in 76% yield, together with a small quantity (12%), of the other regioisomer. The benzyl groups were removed by hydrogenolysis to give compound **(240)** and the exposed hydroxyl groups at 1-, 4- and 5-positions were phosphorylated with tetrabenzylpyrophosphate in the presence of *n*-butyl lithium. Deprotection of the benzylphosphate and isopropylidene protective groups took place using bromotrimethylsilane in dichloromethane followed by the addition of water to give DL-6-deoxy-Ins(1,4,5)$P_3$ (**241** in Figure 62). Ley and coworkers have also made DL-6-deoxy-6-*C*-methyl-Ins(1,4,5)$P_3$ by the same route, but using lithium dimethyl(cyano)copper (I) to open the epoxide in a nucleophilic fashion to give a product which was treated in the same way to obtain the deprotected phosphate. Only DL-6-*O*-methyl-Ins(1,4,5)$P_3$ has been evaluated, which was found to be a weak agonist, [427] some 200-fold weaker than Ins(1,4,5)$P_3$ for $Ca^{2+}$ release with an $EC_{50}$ value of 65μM.



D-6-deoxy-Ins(1,4,5)$P_3$ **(237)**

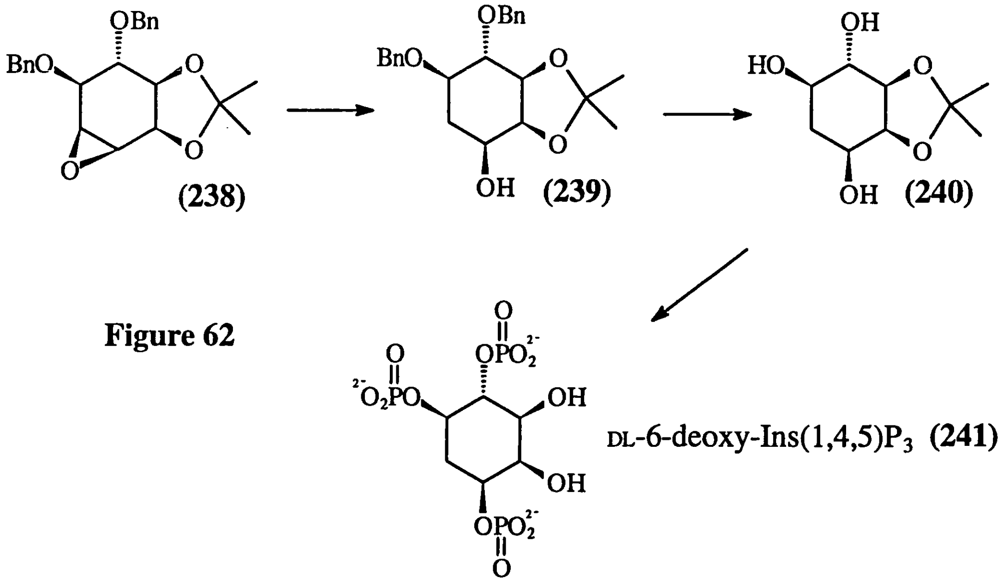


**Figure 62**

DL-6-deoxy-Ins(1,4,5)$P_3$ **(241)**

The biological data for D-6-deoxy-Ins(1,4,5)$P_3$ demonstrated the importance of the 6-hydroxyl group in binding to the Ins(1,4,5)$P_3$ receptor and is recognised by the enzymes 3-kinase and 5-phosphatase. D-6-Deoxy-Ins(1,4,5)$P_3$ was a full agonist for $Ca^{2+}$-release in permeabilised SH-SY5Y neuroblastoma cells with an $EC_{50}$ value of 6.4μM, which was 70-fold less than for Ins(1,4,5)$P_3$ ($EC_{50}$ value of 0.09μM). This difference indicated the importance of the 6-hydroxyl group in binding to the Ins(1,4,5)$P_3$ receptor. D-6-Deoxy-Ins(1,4,5)$P_3$ inhibited [$^{32}$P]Ins(1,4,5)$P_3$ dephosphorylation by 5-phosphatase from HEG with a $K_i$ value of 76μM. [453] The $K_m$ value for Ins(1,4,5)$P_3$ was found to be 40μM [427] and thus D-6-deoxy-Ins(1,4,5)$P_3$ had an apparent 2-fold weaker affinity for 5-phosphatase than Ins(1,4,5)$P_3$. This result agrees with the work carried out by Polokoff and coworkers [427] in which the minimum structural requirements for substrate hydrolysis by 5-phosphatase (from aortic smooth muscle) was suggested to be a free hydroxyl at the 6-position and phosphate groups at the 1-, 4- and 5-positions. The 6-hydroxyl group may provide a catalytic role in the mechanism of 5-phosphatase dephosphorylation in a similar manner to the synthetic 2- and 6-deoxy analogues of Ins(1)P and their interaction with *myo*-inositol monophosphatase. [454]

The 3-kinase from CRBHS showed far greater selectivity than the less specific 5-phosphatase enzyme. D-6-Deoxy-Ins(1,4,5)$P_3$ inhibited the phosphorylation of [$^3$H]Ins(1,4,5)$P_3$ [$K_i$ value of 5.7μM; Ins(1,4,5)$P_3$ had $K_i$ value of 3.2μM]. Thus the analogue binds very well to the 3-kinase but increasing the steric bulk at the 6-position (by methylation) decreased its affinity, ($IC_{50}$ value of 319μM). However, D-6-deoxy-Ins(1,4,5)$P_3$ mobilised $Ca^{2+}$ ions with different kinetics to Ins(1,4,5)$P_3$ and is probably a substrate for the enzyme.

In conclusion, the 6-hydroxyl group, while not essential for $Ca^{2+}$-release, certainly has a role in binding to the receptor, or fixing the conformation of the phosphate groups of Ins(1,4,5)$P_3$.

### 4.7.1 D-3-Deoxy, D-2,3-Dideoxy and D-2,3,6-Trideoxy Ins(1,4,5)$P_3$

The three deoxy compounds were all synthesised from the same starting material, L-quebrachitol. [455] L-Quebrachitol (**169** in Figure 63) was treated with 2-methoxypropene in the presence of camphorsulphonic acid and DMF to form the 3,4:5,6-di-*O*-isopropylidene derivative (**242**). The sodium alkoxide was formed at the 1-position followed by the addition of carbon disulphide and methyl iodide to provide the corresponding *S*-methyldithiocarbonate, which was deoxygenated using *n*-tributyltin hydride in the presence of the radical initiator azobisisobutyronitrile (AIBN) in toluene to give the D-3-deoxy derivative (**243**) (*myo*-inositol numbering). The methyl and isopropylidene groups were then removed using boron tribromide to give D-3-deoxy-*myo*-inositol (**244**) [(-)-viburnitol]. The D-3-deoxy derivative was treated with 2-methoxypropene and catalytic camphorsulphonic acid in DMF to give a mixture of D-3-deoxy-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol and the 1,2:5,6-regioisomer. Benzylation of the mixture gave the 6-*O*-benzyl (**245**) and 4-*O*-benzyl derivatives. The *trans*-isopropylidene acetal was removed using a catalytic amount of HCl and the two hydroxyl groups were benzoylated, at which stage the regioisomers were separated by chromatography, which provided D-4,5-di-*O*-benzoyl-6-*O*-benzyl-3-deoxy-1,2-*O*-isopropylidene-*myo*-inositol (**246**) for further manipulation. The *cis*-1,2-*O*-isopropylidene acetal was removed under acidic conditions [1M aqueous HCl, methanol/THF (4:1)], in order to expose the *cis*-1,2-hydroxyl. Selective benzoylation using benzoyl chloride/DMAP/pyridine, 0°C, for 28h, provided the 1,4,5-tri-*O*-benzoyl derivative (**247**) in 91% yield. This intermediate was used to synthesise all three deoxy-derivatives.

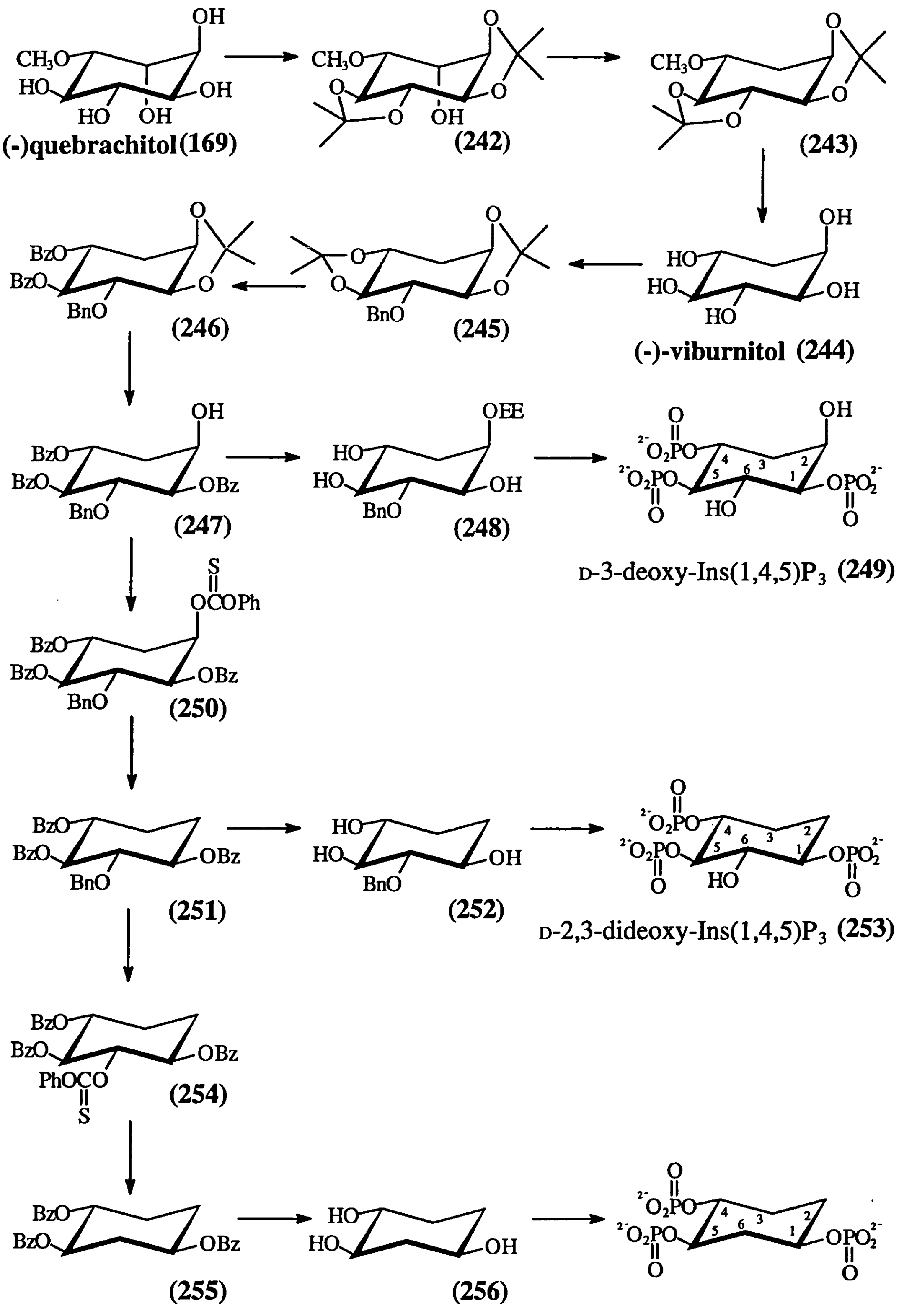



**Figure 63** D-2,3,6-trideoxy-Ins(1,4,5)P$_3$ **(257)**

First, D-3-deoxy-*myo*-inositol 1,4,5-trisphosphate **(249)**, D-3-deoxy Ins(1,4,5)P$_3$, was synthesised by protecting the 2-position as the ethoxyethyl ether to give **(248)** using ethyl vinyl ether and pyridinium toluene-*p*-sulphonate, the benzoyl groups were removed using methanolic potassium carbonate to expose the hydroxyl groups at the

1-, 4- and 5-positions in 94% yield. Phosphorylation using tetrabenzylpyrophosphate and sodium hydride in DMF gave the totally protected phosphorylated compound in 50% yield. Hydrogenation using palladium on carbon, followed by dissolution in water, provided the title compound **(249)** which was isolated as the sodium salt in 56% yield.

Second, D-2,3-dideoxy-*myo*-inositol 1,4,5-trisphosphate **(253)**, D-2,3-dideoxy Ins(1,4,5)$P_3$ was synthesised using the same the intermediate **(247)**. The 2-hydroxyl group was set-up for deoxygenation using the phenoxythiocarbonyl derivative **(250)**, by treatment with phenyl chlorothiocarbonate in the presence of DMAP. Compound **(250)** was treated with tributyltin hydride and AIBN to give the 2,3-dideoxy derivative **(251)**. Debenzoylation as for the 3-deoxy Ins(1,4,5)$P_3$, followed by phosphorylation and deprotection, gave D-2,3-dideoxy Ins(1,4,5)$P_3$ **(253)**.

Third, D-2,3,6-trideoxy Ins(1,4,5)$P_3$ **(257)** was synthesised from the 2,3-dideoxy derivative **(251)**. Debenzylation of compound **(251)**, exposed the 6-hydroxyl which was treated with phenyl chlorothiocarbonate in the presence of DMAP to give **(254)** and deoxygenation as before gave the protected D-2,3,6-trideoxy-*myo*-inositol derivative **(256)**. The benzoate groups were removed with methanolic potassium carbonate and phosphorylated as for the other two deoxy derivatives to give D-2,3,6-trideoxy Ins(1,4,5)$P_3$ **(257)**.

The analogues D-3-deoxy Ins(1,4,5)$P_3$ and D-2,3-dideoxy Ins(1,4,5)$P_3$ were full agonists for $Ca^{2+}$-release at the Ins(1,4,5)$P_3$ receptor from permeabilised SH-SY5Y neuroblastoma cells with $EC_{50}$ values of 155.7 and 185.7nM respectively, which was only 3-fold less than Ins(1,4,5)$P_3$ with had an $EC_{50}$ value of 52.1nM. However, D-2,3,6-trideoxy Ins(1,4,5)$P_3$ was a poor agonist for $Ca^{2+}$-release with an $EC_{50}$ value > 10,000nM. This evidence further identifies the critical role of the 6-hydroxyl in binding to the Ins(1,4,5)$P_3$ receptor whilst the 2- and 3-positions appear to be less important from this data.

D-3-Deoxy Ins(1,4,5)$P_3$ and D-2,3-dideoxy Ins(1,4,5)$P_3$ were evaluated for HEG 5-phosphatase and CRBHS 3-kinase activity. [456] Both D-3-deoxy Ins(1,4,5)$P_3$ and D-2,3-dideoxy Ins(1,4,5)$P_3$ were found to be substrates for 5-phosphatase displacing [$^3$H]Ins(1,4,5)$P_3$ with $K_i$ values of 11.5 and 16.5μM. These values are very similar to those found by Hirata and coworkers [457] for DL-2-deoxy Ins(1,4,5)$P_3$ which had a value of 12.9μM and was also a substrate. The 3-kinase enzyme is substrate specific and both D-3-deoxy and D-2,3-dideoxy Ins(1,4,5)$P_3$ were found to be resistant to 3-kinase activity as predicted with $K_i$ values of 25μM and 19μM respectively.

In summary, the deoxy derivatives have been useful in demonstrating the role of the 6-hydroxyl group in binding to the receptor and the less critical roles of the 2- and 3-hydroxyl groups for this purpose. The 6-deoxy analogue is also resistant to 5-phosphatase indicating a role for the 6-hydroxyl group in 5-phosphatase-catalysed dephosphorylation.

**4.8 Other Analogues**

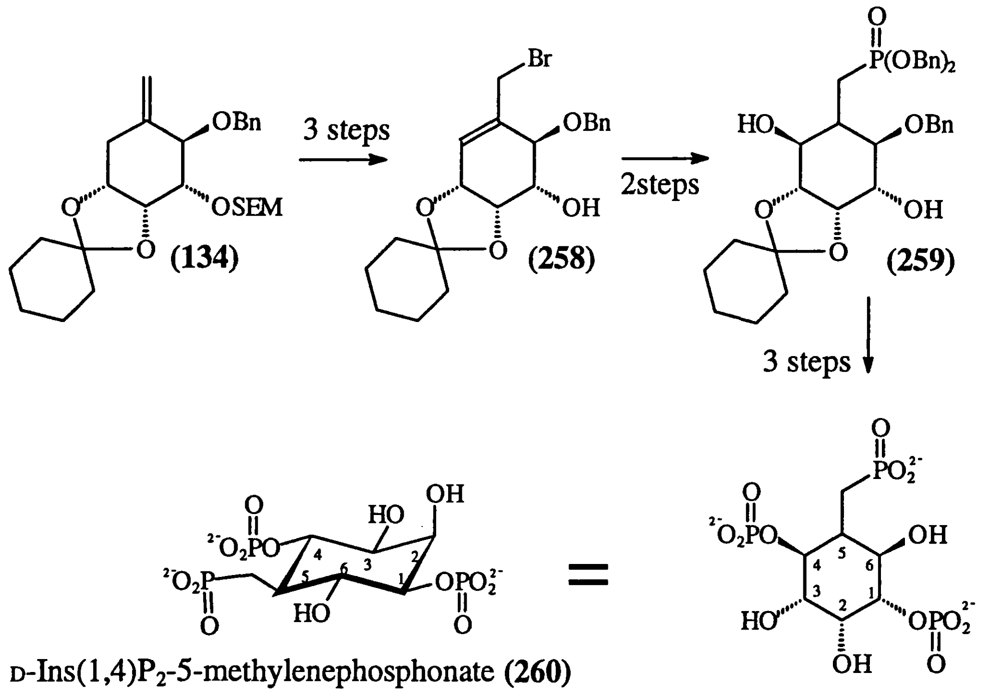
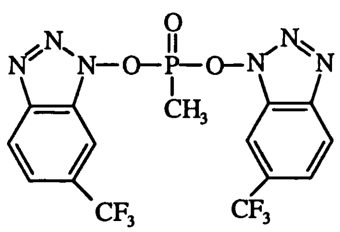



**Figure 64**

Several other analogues have been prepared and evaluated, two of which are phosphonates. First, the 5-methylenephosphonate analogue of Ins(1,4,5)$P_3$ (**260** in Figure 64) was prepared from the commercially available chiral starting material (-)-quinic acid [458] and second, DL-*myo*-inositol 1,4-bisphosphate-5-methylphosphonate (**262** in Figure 65) was prepared from intermediate (**54**). [459] The 5-methylenephosphonate was prepared from intermediate (**134**), which was also used by Falck and coworkers to prepare D-Ins(1,4,5)$P_3$. [403] The 2-(trimethylsilyl)ethoxymethyl ether (SEM), protective group was removed with tetrabutylammonium fluoride in (hexamethylphosphoric triamide) HMPA at 100°C under anhydrous conditions. Kinetically controlled addition of phenylselenyl bromide across the alkene provided the anti-Markovnikov adduct. This intermediate underwent smooth oxidative elimination to provide the endocyclic allylic bromide to give compound **(258)** in 95% yield. Michaelis-Becker phosphorylation of the allylic bromide using excess sodium dibenzyl phosphite in the presence of 18-crown-6 in toluene, followed by hydroboration of the alkene with *m*CPBA, provided compound **(259)** as a single isomer in 95% yield. The 1- and 4-phosphate groups were introduced using the phosphite method [bis(benzyloxy)diisopropylaminophosphine and 1*H*-tetrazole] followed by oxidation with *m*CPBA to furnish the fully protected phosphate. Hydrogenation over palladium on carbon followed by acid hydrolysis of the 2,3-*O*-cyclohexylidene acetal gave the 5-methylenephosphonate analogue **(260)** in 67% yield, isolated as the sodium salt.

The phosphonate analogue elicited contraction of permeabilised bovine tracheal smooth muscle, with a 5-10 fold lower potency than D-Ins(1,4,5)$P_3$. The compound was also 5-phosphatase resistant, and in bovine adrenal microsomes was equally potent to D-Ins(2,4,5)$P_3$, and about one-fifth as active as D-Ins(1,4,5)$P_3$.

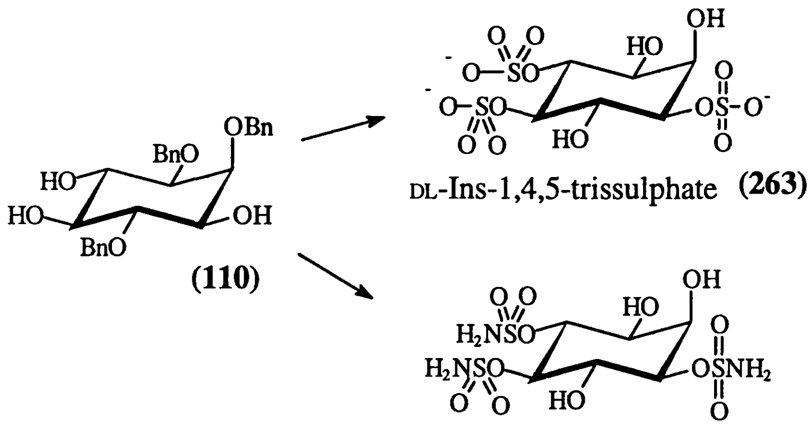

**(54)** DL-Ins(1,4)$P_2$-5-methylphosphonate **(262)**



**(261)**

**Figure 65**

The 5-methylphosphonate analogue **(262)** was prepared by van Boom and coworkers [459] from the 1,4-bisphosphate derivative **(54)**. Thus, phosphonylation of the 5-hydroxyl with excess bis[6-(trifluoromethyl)benzotriazol-1-yl]-methylphosphonate **(261)** gave, after 15min at 20°C, the putative [6-(trifluoromethyl)benzotriazol-1-yl]-methyl phosphonate intermediate. This was treated *in situ* with benzyl alcohol and *N*-methylimidazole to give after 1h at 20°C, the fully protected Ins(1,4,5)$P_3$ analogue as a mixture of diastereoisomers. Hydrogenolysis gave the methylphosphonate analogue which was isolated as the sodium salt. Biological evaluation initially indicated that the racemic analogue was an antagonist at the Ins(1,4,5)$P_3$ receptor from permeabilised platelets. However, this finding has not been confirmed by any biological publication.



(110)

DL-Ins-1,4,5-trissulphate (263)

**Figure 66** DL-Ins-1,4,5-trissulphonamide **(264)**

The three phosphates of Ins(1,4,5)$P_3$ have been replaced by sulphates and sulphonamides. [460] DL-1,2,4-Tri-*O*-benzyl-*myo*-inositol **(110)** (Figure 66) was treated with triethylamine-sulphur trioxide complex at 50°C for 16h in DMF, followed by hydrogenolysis over palladium on carbon in DMF-water (4:1) to give *myo*-inositol 1,4,5-trissulphate **(263)** in 86% yield. The 1,4,5-trissulphamoylated derivative was prepared by treating DL-1,2,4-tri-*O*-benzyl-*myo*-inositol **(110)** and sulphamoyl chloride with sodium hydride in DMF at 0°C for 2h. Debenzylation by hydrogenolysis provided *myo*-inositol 1,4,5-trissulphamate **(264)** in 84% yield from the starting triol. Neither of these compounds displayed any binding to the Ins(1,4,5)$P_3$ receptor from platelets. 3-Azido-3-deoxy-*myo*-inositol 2,4,5-trisphosphate has also been prepared (not shown) and did not show any interesting biological activity for 3-kinase nor $Ca^{2+}$-release. However, it was a substrate for 5-phosphatase inhibiting the dephosphorylation of [$^3$H]Ins(1,4,5)$P_3$ with a $K_i$ value of 36.3μM. [456]

Benzene 1,2,4-trisphosphate [461] has been prepared by phosphorylating benzene 1,2,4-triol with dibenzylphosphochloridate in the presence of *N,N*-diisopropylethylamine. The benzyl groups were removed using 70% trifluoroacetic acid in dichloromethane, and purified by reverse phase HPLC, to give pure benzene 1,2,4-trisphosphate. The analogue interacted with the Ins(1,4,5)$P_3$ receptor but did not release $Ca^{2+}$. The analogue showed inhibition of 5-phosphatase with an $IC_{50}$ value of 32μM (from adrenal cortex microsomes) and an $IC_{50}$ value for 3-kinase inhibition of 6.1μM (from adrenal cortex cytosol).

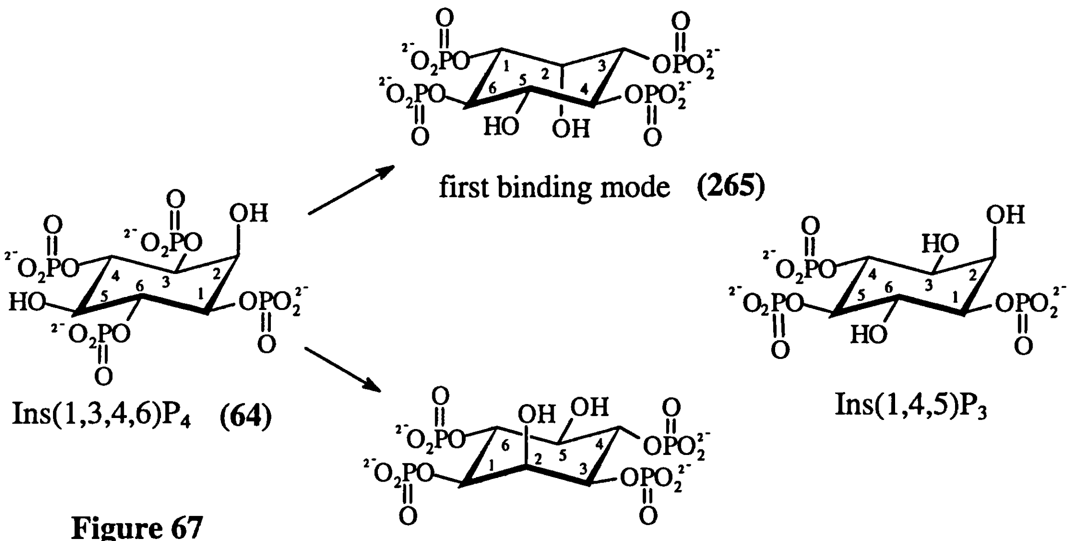
## 4.9 Partial Agonists at the Ins(1,4,5)$P_3$ Receptor

The term *partial agonist* was first used in order to provide an explanation for agonists which display a large range of intrinsic activities at the same receptor depending on the conditions and model used. [462] The term *efficacy* is used to distinguish between the agonist binding to the receptor measured by its equilibrium constant, and the ability of the agonist to produce a response (for example $Ca^{2+}$-release) once it has bound. For example, in a specific tissue, two different agonists acting on the same receptor may generate different maximal responses from the same tissue. At 100% receptor occupancy one agonist may produce a smaller effect than another agonist and in parallel with this idea arrived the concept of spare receptors. A high efficacy agonist will produce a maximum response when it occupies only a small fraction of receptors, there are in other words, 'spare receptors'. However, an agonist with low efficacy will not produce a maximum response even when all the receptors are occupied. The difference between agonists, partial agonists and antagonists may become a matter of efficacy. Antagonists have zero efficacy, partial agonists have low variable efficacy and agonists have high efficacy. In this last section partial agonists will be discussed with respect to $Ca^{2+}$-release and finally, antagonists, which may be useful as tools for pharmacological interaction.

### 4.9.1 Ins(1,3,4,6)$P_4$

The naturally occurring *meso*-compound *myo*-inositol 1,3,4,6-tetrakisphosphate, **(64)** Ins(1,3,4,6)$P_4$ was synthesised by deprotection of 2,5-di-*O*-benzyl-1,3,4,6-tetra[di(2-cyanoethoxyphospho)]-*myo*-inositol, first by hydrolysis of the base-labile cyanoethoxy groups followed by hydrogenation of the benzyl groups at the 2- and 5-positions. It was found that Ins(1,3,4,6)$P_4$ mediated $Ca^{2+}$-release from the Ins(1,4,5)$P_3$ receptor of SH-SY5Y human neuroblastoma cells. However, **(64)** was also found to be a partial agonist with an $EC_{50}$ value of 5.92μM compared to Ins(1,4,5)$P_3$ with an $EC_{50}$ value of 0.12μM. [216] Thus, Ins(1,3,4,6)$P_4$ is 50-fold less potent at $Ca^{2+}$-release and only released a maximum of 80-90% of the $Ca^{2+}$ pool that is normally released by Ins(1,4,5)$P_3$. Furthermore, Ins(1,3,4,6)$P_4$ behaves a full agonist at other Ins(1,4,5)$P_3$ receptors. The reason for the observed partial agonism of Ins(1,3,4,6)$P_4$ at SH-SY5Y human neuroblastoma cells and more recent in rat brain microsomes [431] is not obvious. By further modification of this structure one will hopefully produce an analogue with even lower efficacy and therefore closer to an antagonist.

The important functional aspects for $Ca^{2+}$-release are a vicinal D-4,5-bisphosphate and an adjacent 6-hydroxyl to increase the binding at the Ins(1,4,5)$P_3$ receptor. A phosphate group at the 1-position further enhances binding to the receptor. The *meso*-Ins(1,3,4,6)$P_4$ does not have a free 6-hydroxyl or a D-4,5-bisphosphate. However, the molecule may bind to the Ins(1,4,5)$P_3$ receptor in one of two orientations which maximise similarity to Ins(1,4,5)$P_3$ (Figure 67). In the first binding mode **(265)** the 4-, 1- and 6-phosphate groups may represent a pseudo D-1,4,5-trisphosphate with the 5-hydroxyl mimicking the 6-hydroxyl group of Ins(1,4,5)$P_3$. The pseudo 2-position is phosphorylated and equatorial but this does not affect the $Ca^{2+}$-releasing activity as exemplified by the activity of racemic Ins(1,2,4,5)$P_4$ and *scyllo*-Ins(1,2,4,5)$P_4$. The pseudo 3-position of Ins(1,3,4,6)$P_4$ is now axial and may play a part in the partial agonist behaviour. In the second orientation **(266)**, the pseudo D-1,4,5-trisphosphate moiety is provided by the 3-, 6- and 1-phosphates from Ins(1,3,4,6)$P_4$ with an equatorial 3-hydroxyl [5-hydroxyl from Ins(1,3,4,6)$P_4$] and the pseudo 6-position is occupied by the axial 2-hydroxyl of Ins(1,3,4,6)$P_4$. It is difficult to determine which orientation or both is involved in this partial agonist behaviour.



first binding mode **(265)**

Ins(1,3,4,6)$P_4$ **(64)**

Ins(1,4,5)$P_3$

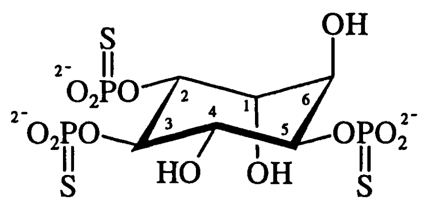
second binding mode **(266)**

**Figure 67**

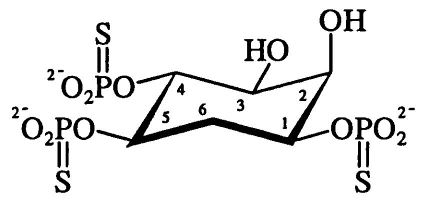
Ins(1,3,4,6)$P_4$ was also found to be a poor inhibitor of 3-kinase from CRBHS with a $K_i$ value of 150μM. However, it was found to be a substrate, and like other tetrakisphosphates tested, Ins(1,3,4,6)$P_4$ had a high affinity for 5-phosphatase from HEG, and inhibited [$^3$H]Ins(1,4,5)$P_3$ dephosphorylation with a $K_i$ value of 7.7μM. [422]

### 4.9.2 L-*chiro*-Ins(2,3,5)PS$_3$ and D-6-deoxy-Ins(1,4,5)PS$_3$

L-*chiro*-Inositol 2,3,5-trisphosphorothioate (**267** Figure 68) [L-*chiro*-Ins(2,3,5)PS$_3$] was synthesised in a similar manner to L-*chiro*-Ins(2,3,5)P$_3$ described in section 4.4.1. Essentially, L-1,4,6-tri-*O*-benzoyl-*chiro*-inositol was phosphitylated with bis(2-cyanoethoxy)diisopropylaminophosphine and 1*H*-tetrazole, followed by sulphoxidation with sulphur in pyridine. Work up and purification by flash chromatography gave the protected trisphosphorothioate in 90% yield. All the protective groups were removed by sodium in liquid ammonia reduction followed by final purification by ion exchange chromatography to provide L-*chiro*-Ins(2,3,5)PS$_3$ **(267)** in 70% yield. [463] D-6-Deoxy-*myo*-inositol 1,4,5-trisphosphorothioate, **(268)**, D-6-deoxy-Ins(1,4,5)PS$_3$, was synthesised by phosphitylating D-2,3-*O*-cyclohexylidene-6-deoxy-*myo*-inositol [464] using bis(benzyloxy)diisopropylaminophosphine and 1*H*-tetrazole, followed by sulphoxidation with sulphur in pyridine. The phosphorothioate protective groups were removed using sodium in liquid ammonia and the *cis*-2,3-*O*-cyclohexylidene acetal was removed using H$^+$ Dowex ion exchange resin. The residue was purified by ion exchange chromatography over DEAE Sephadex-A25 resin and eluted with TEAB to give D-6-deoxy-Ins(1,4,5)PS$_3$ **(268)**.



L-*chiro*-Ins(2,3,5)PS$_3$ **(267)**



D-6-deoxy-Ins(1,4,5)PS$_3$ **(268)**

**Figure 68**

The binding and Ca$^{2+}$-mobilising properties of L-*chiro*-Ins(2,3,5)PS$_3$ and D-6-deoxy-Ins(1,4,5)PS$_3$ with the Ins(1,4,5)P$_3$ receptor from permeabilised SH-SY5Y human neuroblastoma cells were investigated. It was found using radioligand assays, that both analogues were able to compete with [$^3$H]Ins(1,4,5)P$_3$ for specific sites on adrenal cortical membranes, albeit weaker than Ins(1,4,5)P$_3$. Both these analogues could compete at the Ins(1,4,5)P$_3$ receptor with $K_d$ values of 500nM for L-*chiro*-Ins(2,3,5)PS$_3$ and 5300nM for D-6-deoxy-Ins(1,4,5)PS$_3$, whereas Ins(1,4,5)P$_3$ had a $K_m$ value of 6.5nM. The intrigue of these analogues was that they showed low efficacy, releasing only 34% of the Ca$^{2+}$ with respect to Ins(1,4,5)P$_3$ for L-*chiro*-Ins(2,3,5)PS$_3$, and 42% for D-6-deoxy-Ins(1,4,5)PS$_3$. Both compounds were able to inhibit the response of Ins(1,4,5)P$_3$-induced Ca$^{2+}$-release with $K_i$ values of 6μM and 33μM respectively for L-*chiro*-Ins(2,3,5)$PS_3$, and D-6-deoxy-Ins(1,4,5)$PS_3$ which were very similar to the $EC_{50}$ values of 5.3μM and 16μM respectively. These two compounds represent analogues with low efficacy and are valuable leads in the synthesis of compounds with zero efficacy and thus zero $Ca^{2+}$-release. By further structural modification both at the phosphate and at the hydroxyl function, a better tool may be found in order to investigate the structure-activity relationships of the Ins(1,4,5)$P_3$ receptor. The reasons why these two compounds show lower efficacy than normal are not clear. However, two features that both these compounds have in common are: first, both analogues are phosphorothioates, with a pseudo D-1,4,5-configuration and second, both have a single structural modification at a hydroxyl group. L-*chiro*-Ins(2,3,5)$PS_3$ has an inverted 3-hydroxyl and D-6-deoxy-Ins(1,4,5)$PS_3$ has a deleted 6-hydroxyl moiety. The substitution of phosphates for phosphorothioates leads to an increase in size, hydrophobicity and charge distribution. One or all of which may participate to produce a response of lower efficacy. Another second messenger, cyclic adenosine monophosphate (cyclic AMP) has been substituted with a phosphorothioate to produce the only known competitive antagonist, $R_p$ adenosine 3',5'-cyclic-monophosphorothioate [465] and $R_p$ adenosine 3',5'-cyclic-monophosphorodithioate [466] of the several hundreds of analogues that have been synthesised. L-*chiro*-Ins(2,3,5)$P_3$ was also found to be a partial agonist in permeabilised platelets where it released only 42.3% of the $Ca^{2+}$-pool that was available to Ins(1,4,5)$P_3$. [463]

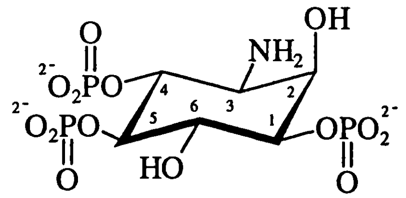
Both these compounds have been tested for resistance to 5-phosphatase from HEG and 3-kinase from CRBHS. It was found that L-*chiro*-Ins(2,3,5)$PS_3$ was the most potent inhibitor of 5-phosphatase with a $K_i$ value of 0.23μM, and was a potent inhibitor of 3-kinase with a $K_i$ value of 0.82μM. [422] D-6-Deoxy-Ins(1,4,5)$PS_3$ was found to be resistant to 3-kinase and 5-phosphatase enzymes with $K_i$ values of 7.9μM and 1.4μM respectively. [422]

### 4.9.3 D-3-Amino-3-deoxy Ins(1,4,5)$P_3$ and *scyllo*-Ins(1,2,4,5)$PS_4$

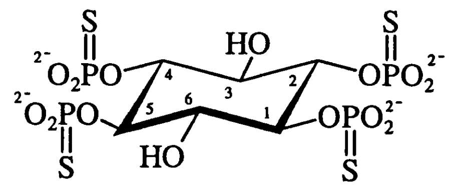
D-3-Amino-3-deoxy-*myo*-inositol 1,4,5-trisphosphate **(269)**, D-3-amino-3-deoxy-Ins(1,4,5)$P_3$ was prepared by Kozikowski and coworkers, [467] and *scyllo*-inositol 1,2,4,5-tetrakisphosphorothioate **(270)**, *scyllo*-Ins(1,2,4,5)$PS_4$, was prepared by D. Lampe (Ph.D thesis 1993). [413]

D-3-Amino-3-deoxy-Ins(1,4,5)$P_3$ was prepared from D-3-azido-3-deoxy-*myo*-inositol. Briefly, D-3-azido-3-deoxy-*myo*-inositol was treated in the same way as for D-3-deoxy-3-fluoro-*myo*-inositol in order to obtain the totally protected phosphorylated D-3-

azido-3-deoxy-*myo*-inositol derivative. Hydrogenation over palladium on carbon under acidic conditions removed all the protective groups and reduced the D-3-azido group to give the D-3-amino-3-deoxy-Ins(1,4,5)$P_3$ **(269)**. *scyllo*-Ins(1,2,4,5)$PS_4$ was prepared from the *meso*-compound 1,4-di-*O*-benzyl-*scyllo*-inositol [the same intermediate that was used to prepare *scyllo*-Ins(1,2,4,5)$P_4$]. Phosphitylation of the four hydroxyl groups using bis(benzyloxy)disopropylaminophosphine followed by sulphoxidation (sulphur in pyridine) gave the totally protected tetrakisphosphorothioate. Deprotection using sodium in liquid ammonia provided *scyllo*-Ins(1,2,4,5)$PS_4$ in 18% yield.



D-3-amino-Ins(1,4,5)$P_3$ **(269)**



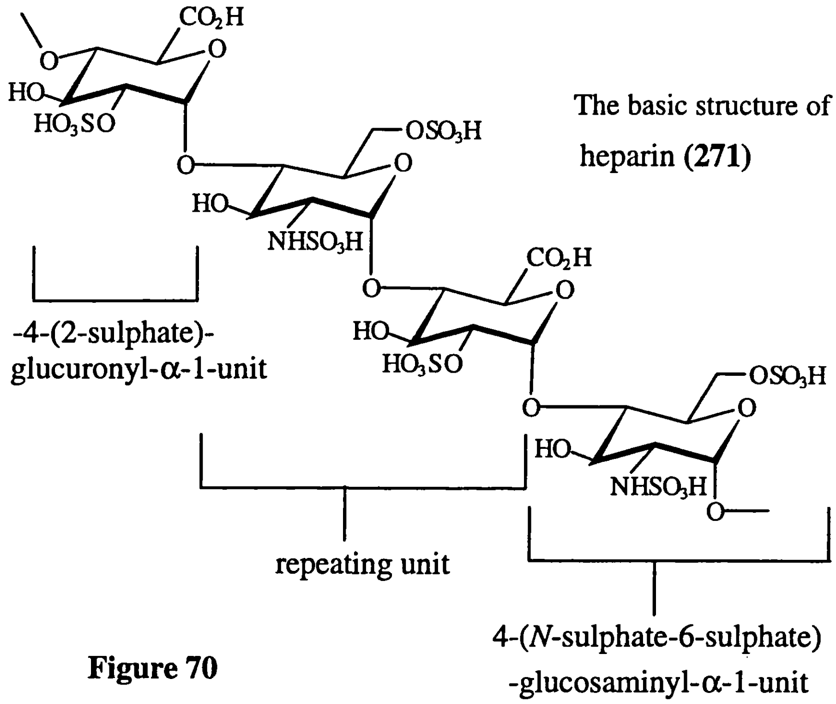
*scyllo*-Ins(1,2,4,5)$PS_4$ **(270)**

**Figure 69**

D-3-Amino-3-deoxy-*myo*-inositol 1,4,5-trisphosphate mobilised $Ca^{2+}$ from saponin-permeabilised SH-SY5Y neuroblastoma cells. The experiments were carried out at three different pH values, 6.8, 7.2 and 7.6. It was found that the $EC_{50}$ values varied both with Ins(1,4,5)$P_3$ and D-3-amino-3-deoxy-Ins(1,4,5)$P_3$. However, at pH 6.8 the $EC_{50}$ value for Ins(1,4,5)$P_3$ was 255nM but the $EC_{50}$ value for D-3-amino-3-deoxy-Ins(1,4,5)$P_3$ was 3676nM and D-3-amino-3-deoxy-Ins(1,4,5)$P_3$ released only 80% of the possible Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool. At pH 7.2 and 7.6 the analogue was a full agonist with $EC_{50}$ values of 1070nM and 1491nM respectively. In addition the analogue was able to fully displace [$^3$H]Ins(1,4,5)$P_3$ from binding sites on rat cerebellum membranes at pH 6.8 and 7.6 indicating that the analogue fully interacted with the Ins(1,4,5)$P_3$ receptor. No data on the interaction of this analogue with the enzymes 3-kinase or 5-phosphatase have been published.

*scyllo*-Ins(1,2,4,5)$PS_4$ was also demonstrated to be a partial agonist in saponin permeabilised SH-SY5Y neuroblastoma cells. *scyllo*-Ins(1,2,4,5)$PS_4$ was able to mobilise 80% of the $Ca^{2+}$ from the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool with an $EC_{50}$ value of 1.6μM. [414] *scyllo*-Ins(1,2,4,5)$PS_4$ displaced [$^3$H]Ins(1,4,5)$P_3$ from binding sites on bovine adrenal cortex membranes with an $IC_{50}$ value of 424.6nM. The compound can only bind in one orientation because it is symmetrical, and the phosphorothioate substitution is critical for partial agonism because *scyllo*-Ins(1,2,4,5)$P_4$ was a full agonist at the same Ins(1,4,5)$P_3$ receptor. *scyllo*-Ins(1,2,4,5)$PS_4$ was also found to be a potent inhibitor of 5-phosphatase from HEG with a $K_i$ value of 0.3μM and of 3-kinase from CRBHS with a $K_i$ value of 56.7μM.

### 4.9.4 The Ins(1,4,5)$P_3$ Receptor Antagonists Heparin and Decavanadate




The basic structure of heparin **(271)**

**Figure 70**

Heparin (Figure 70) is a heteroglycan, containing alternating sulphated units of glucuronic acid and glucosamine in a straight chain of 30-70 glycose units. Heparin is highly acidic due to the sulphate groups and a powerful anticoagulant. Heparin was the first antagonist at the Ins(1,4,5)$P_3$ receptor to be recognised, binding with with reasonable potency resulting in the inhibition of $Ca^{2+}$-mobilisation from several systems, including bovine adrenal cortex with an $IC_{50}$ value of 10μg/ml [456] and 4.5μg/ml in rat liver microsomes. [456] Heparin has been used to purify the Ins(1,4,5)$P_3$ receptor using heparin-agarose affinity chromatography which has been discussed in section 2.6.1. It is easy to speculate that the charged sulphate groups of heparin may interact with the Ins(1,4,5)$P_3$ receptor which is normally responsible for for binding phosphate groups, since *N*-desulphation of heparin decreased the antagonistic activity. [468]

Another molecule that was found to be a competitative antagonist, was decavanadate, which inhibited $Ca^{2+}$-release from intracellular stores with a $K_i$ value of 1.2μM. [469] The molecule is anionic with low specificity, and was able to bind to both the Ins(1,4,5)$P_3$ and Ins(1,3,4,5)$P_4$ recognition sites. Orthovanadate was not a competititive antagonist but tetravanadate has been reported to inhibit phospholipase C. [470]

Table 6 provides a summary of modifications to Ins(1,4,5)$P_3$ both at the phosphate and hydroxyl functions with respect to the Ins(1,4,5)$P_3$ receptor-ligand interaction and 5-phosphatase and 3-kinase inhibition.

**Table 6**

| Position number | Receptor Binding | 3-Kinase Interaction | 5-Phosphatase Interaction |
|---|---|---|---|
| 1 | Large substituents allowed without loss of activity. Deletion decreases activity. | 1-Phosphate is important however not essential. Ins(4,5)$P_2$ is a weak substrate. | 1-Phosphate enhances recognition. Ins(4,5)$P_2$ is a weak substrate. |
| 2 | Bulky groups are tolerated. Hydroxyl group deletion and inversion also tolerated, together with fluoro- and phosphorylated derivatives. | The axial hydroxyl is important for recognition, although 2-deoxy Ins(1,4,5)$P_3$ is a substrate. Inversion of the hydroxyl group reduces affinity 5-fold. Phosphorylation $\rightarrow$ Ins(1,2,4,5)$P_4$ removes recognition. | 2-Deoxy-Ins(1,4,5)$P_3$ is a good substrate. However, substitution with a phosphate gives a good inhibitor. |
| 3 | Deoxy-, fluoro- and amino-groups bind to the receptor. Inversion to give *chiro*-derivative also recognised, but hydrophobic substituents larger than OMe fail to mobilise calcium. | Deoxy-, fluoro- and *chiro*-derivatives are potent inhibitors of 3-kinase. | Deoxy- and fluoro-Ins(1,4,5)$P_3$ are good substrates. *chiro*-Ins(2,3,5)$P_3$ is a potent inhibitor. |
| 4 | No 4-modified compounds have been made. | No 4-modified compounds have been made. | No 4-modified analogues have been made. |
| 5 | 4,5-Bisphosphate is essential for recognition. Ins(1,5)$P_2$ and Ins(1,4)$P_2$ do not bind to receptor. | Ins(1,4,5)$P_3$-5S is slowly phosphorylated by enzyme. | Ins(1,4,5)$P_3$-5S and 5-methylenephosphonate-Ins(1,4,5)$P_3$ are potent inhibitors. |
| 6 | Removal of the hydroxyl reduces affinity 70-fold. Methylation reduces the affinity even further. | 6-Deoxy-Ins(1,4,5)$P_3$ is a substrate. Bulky substituents reduce affinity. | Mechanistic hydroxyl group. Deletion gives an inhibitor. |

# CHAPTER FIVE

# Results And Discussion

## RESULTS AND DISCUSSION

### 5.1 Aims of the Project

The biology of *myo*-inositol phosphates has developed immensely over the last decade. Since the first critical paper which was published in 1983, there have been approximately 500 biological publications per annum from 1987 to the present. However, the number of chemical papers lags behind because of the initial problems encountered with the synthesis of *myo*-inositol phosphates which did not commence until 1987. Thus, there is a need to produce analogues which will disrupt the enzymes 3-kinase and 5-phosphatase and synthesise a full antagonist which will be used to investigate the structure-function requirements of the Ins(1,4,5)$P_3$ receptor.

In the work described in this thesis, fourteen *myo*-inositol phosphate analogues were prepared and fully characterised, thirteen of which were based on *myo*-inositol and one based on the planar benzene ring with phosphates arranged in a pseudo 1,4,5-trisphosphate orientation.

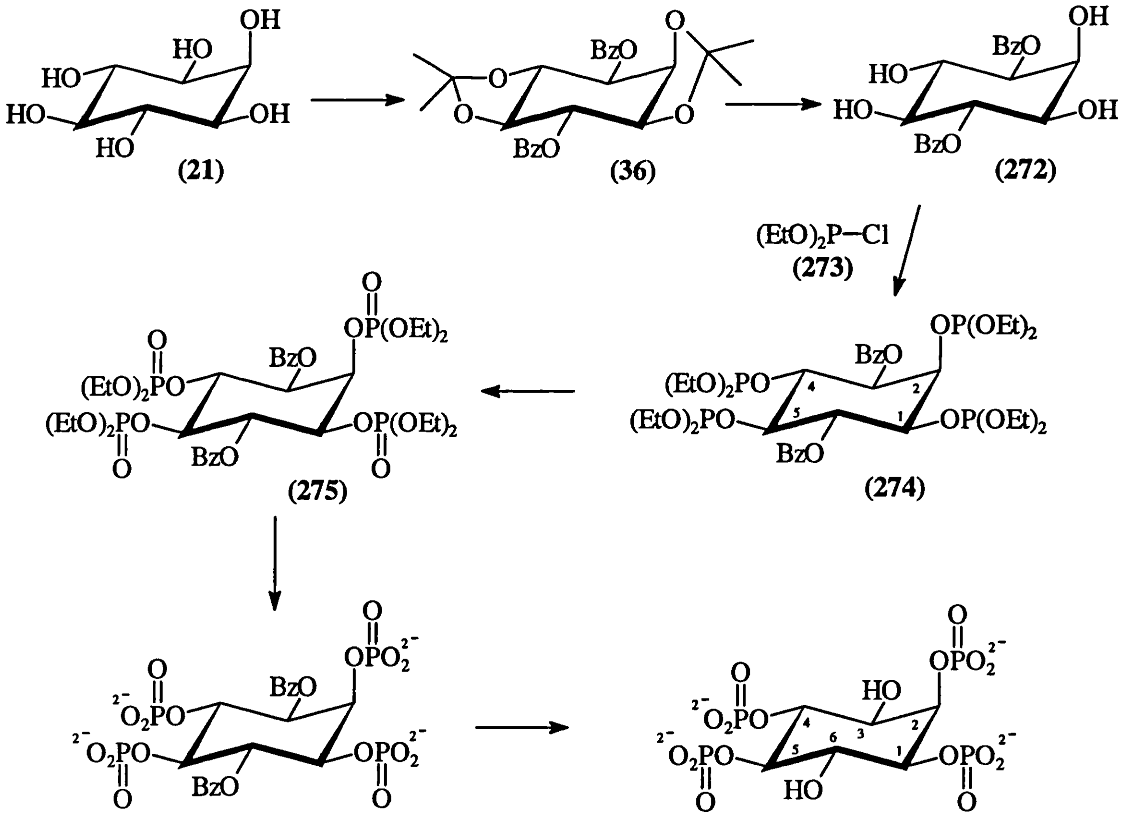
The first target compounds to be synthesised were DL-3,6-di-*O*-benzoyl Ins(1,2,4,5)$P_4$ **(276)** and DL-Ins(1,2,4,5)$P_4$, **(152)** which were initially made in racemic form. The latter compound was then resolved from a suitable intermediate in order to provide D- and L-enantiomers of Ins(1,2,4,5)$P_4$, once the biological data for the racemic mixture had been evaluated. D-Ins(1,2,4,5)$PS_4$ was also synthesised, in order to investigate if it had properties similar to those of *scyllo*-Ins(1,2,4,5)$PS_4$, which is a partial agonist at the Ins(1,4,5)$P_3$ receptor which was discussed in section 4.9.3.

Methylation of the hydroxyl groups at the 2- and 5-positions of Ins(1,3,4,6)$P_4$ indicated that these positions were important for binding at the Ins(1,4,5)$P_3$ receptor because the resulting compound did not interact with the receptor. The corresponding 2,5-di-*O*-methyl Ins(1,3,4,6)$PS_4$ analogue was also prepared in order to investigate its interaction with the enzymes 3-kinase and 5-phosphatase. The phosphorothioate analogue Ins(1,3,4,6)$PS_4$ was prepared to see if it was a partial agonist like Ins(1,3,4,6)$P_4$, in SH-SY5Y human neuroblastoma cells.

The structure of Ins(1,3,4,6)$P_4$ was then dissected to give two compounds, in order to investigate which part of the molecule provided the partial agonist effect present in SH-SY5Y human neuroblastoma cells. The two analogues D-Ins(1,4,6)$P_3$ and L-Ins(1,3,4)$P_3$ have the Ins(1,4,5)$P_3$ motif, but the orientations of certain hydroxyl groups were different (this was discussed in section 4.4.5). Only one molecule was synthesised by the author, namely Ins(1,4,6)$P_3$. First, DL-Ins(1,4,6)$P_3$ was made in order to investigate its $Ca^{2+}$-releasing properties together with its phosphorothioate counterpart. A route was then undertaken in order to prepare both the D- and L-enantiomers of Ins(1,4,6)$P_3$ together with D-Ins(1,4,6)$PS_3$, because DL-Ins(1,4,6)$PS_3$ was found to be a partial agonist with low efficacy in rabbit platelets.

Finally, the analogue based on the benzene ring, benzene 1,2,4-trisphosphate **(360)**, was synthesised as a potential inhibitor of 5-phosphatase and 3-kinase. Later it was also tested on the p85 monoclonal antibody of immunoprecipitated PtdIns 3-kinase and was the first Ins(1,4,5)$P_3$-like molecule to inhibit this enzyme.

## 5.2 Synthesis of DL-3,6-Di-*O*-Benzoyl Ins(1,2,4,5)$P_4$ and DL-Ins(1,2,4,5)$P_4$




DL-3,6-Di-*O*-benzoyl-Ins(1,2,4,5)$P_4$ **(276)**

DL-Ins(1,2,4,5)$P_4$ **(152)**

**Figure 71**

The aim of preparing DL-3,6-di-*O*-benzoyl Ins(1,2,4,5)$P_4$ **(276)** and DL-Ins(1,2,4,5)$P_4$ **(152)** (Figure 71) was first, to establish the effect of a phosphate group at the 2-position and second, to investigate the effect of blocking the 3- and 6- hydroxyl groups on $Ca^{2+}$-release, and establish what effect these compounds have on the Ins(1,4,5)$P_3$ metabolising enzymes, 3-kinase and 5-phosphatase.

DL-1,4-Di-*O*-benzoyl-*myo*-inositol **(272)** was the phosphorylation precursor which was used to synthesise **(276)** and **(152)**. This tetrol was synthesised in a three step two pot reaction. Thus, DL-3,6-di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(36)** was prepared in 30% yield using the method developed by Gigg and coworkers, in the following way. [344] A mixture of *myo*-inositol **(21)**, 2,2-dimethoxypropane and a catalytic amount of toluene-*p*-sulphonic acid was stirred in DMF at 100°C for 2h. The mixture was cooled to 0°C and pyridine was added to the mixture followed by benzoyl chloride. Stirring was continued for a further 2h and the resulting thick yellow-brown soup was filtered into a sinter funnel. The mixture was then washed with pyridine to remove any unreacted benzoyl chloride and water to dissolve the pyridinium hydrochloride. The remaining solid was then washed with acetone to remove water and contaminating organic material, and finally ether to give a compact white powder. The crude compound was insoluble in the washing solvents and pure enough for the synthesis of DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(37)**, described in section 5.3.1. However, a small quantity of **(36)** was recrystallised from hot DMF for the $^1$H NMR, melting point and synthesis of the intermediate **(272)**. DL-3,6-Di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(36)** was unusual because it was highly insoluble in organic solvents at room temperature. The $^1$H NMR spectrum was poorly resolved in $d_6$-DMSO, but surprisingly a small quantity (~ 5mg) was soluble in 0.5ml of $CDCl_3$. The melting point for compound **(36)** was 328-330°C, and unusually high for a *myo*-inositol derivative. The $^1$H NMR spectrum for compound **(36)** showed downfield shifts [in comparison to **(37)**] for C-6-H and C-3-H at $\delta$ = 5.61 (dd, J = 6.78 and 11.17Hz) and $\delta$ = 5.43 (dd, J = 4.21 and 10.63Hz), respectively, due to carbonyl deshielding from the benzoyl group.

The washings provided several other compounds, including DL-3,4-di-*O*-benzoyl-1,2:5,6-di-*O*-isopropylidene-*myo*-inositol and DL-3,4,5,6-tetra-*O*-benzoyl-1,2-*O*-isopropylidene-*myo*-inositol. Recently, these were separated in the following manner. [471] The combined filtrate and washings were partitioned between water and dichloromethane, the organic layer was separated and concentrated *in vacuo*. The solubility of DL-3,4-di-*O*-benzoyl-1,2:5,6-di-*O*-isopropylidene-*myo*-inositol in methanol was much higher than DL-3,4,5,6-tetra-*O*-benzoyl-1,2-*O*-isopropylidene-*myo*-inositol. Thus, the mixture was washed with methanol and the two compounds were then isolated in 28% and 13% yield respectively.

The relatively insoluble DL-3,6-di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(36)** was suspended in 80% acetic acid and heated under reflux for 30min, after which time a clear solution was seen. The mixture was cooled, and poured into an ice-water mixture to cause precipitation. The solid was filtered off and washed thoroughly with ether to give DL-1,4-di-*O*-benzoyl-*myo*-inositol **(272)**. Compound **(272)** was insoluble in all protic solvents, including hot ethanol and methanol. However, precipitation from DMF-water provided the pure tetrol in 93% yield which had a melting point of 253°C. The $^{1}H$ NMR in $d_6$-DMSO indicated that the 1- and 4-positions were protected and at positions 2-, 3-, 5- and 6-, the ring hydrogens coupled to their respective hydroxyl groups. This tetrol was also synthesised by Meek and coworkers, [393] but full experimental details were not given.

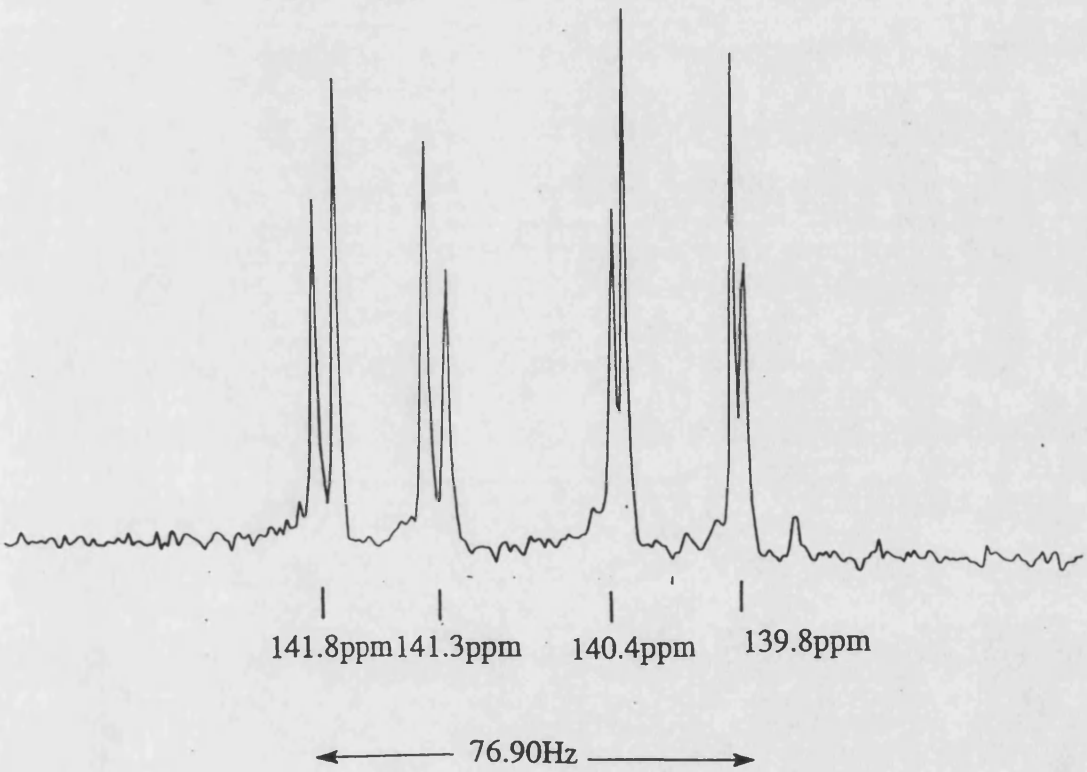



**Figure 72**

The cheap commercially available P(III) reagent diethoxychlorophosphine **(273)**, ($\delta_P$ = +167ppm), was used to phosphitylate DL-1,4-di-*O*-benzoyl-*myo*-inositol **(272)**. The tetrol was dissolved in a mixture of dry DMF and dry *N,N*-diisopropylethylamine and stirred under nitrogen. The mixture was cooled to 0°C and diethoxychlorophosphine was added slowly over a 5min period and stirred for 1h at room temperature. At this stage, the $^{31}$P NMR spectrum of **(274)** operating at 36.2MHz with a sweep width of 2500KHz, showed eight peaks resulting from two $^{5}J_{PP}$ AB coupling systems centered around $\delta$ = +141.8 and +141.3ppm (for the 4,5-positions) and +140.4 and 139.8ppm (for the 1,2-positions) for each doublet of the AB coupling pattern. This AB spectrum is shown in Figure 72 which demonstrated phosphitylation of a pair of vicinal diols at the 1,2-positions, where $^{5}J_{PP}$ = 1.83Hz and $^{5}J_{PP}$ = 3.66Hz for the 4,5-positions.

Oxidation of the 1,2,4,5-tetrakisphosphite with *t*-butylhydroperoxide provided DL-3,6-di-*O*-benzoyl-1,2,4,5-tetrakis(diethoxyphospho)-*myo*-inositol **(275)** and destroyed the two AB patterns shifting the phosphate signals upfield to -1.53, -2.11, -2.18 and -2.49ppm in the $^{31}$P-$^{1}$H-decoupled spectrum. Surprisingly the tetrakisphosphate was isolated as a crystalline solid, m.p. 122-123°C (from ethyl acetate-hexane).

The 400MHz $^{1}$H NMR spectrum of crystalline **(275)** resolved the *myo*-inositol ring hydrogens which overlapped at 270 MHz. The triplet at $\delta$ = 5.90 was identified as C-6-H whilst the two triple doublets at $\delta$ = 5.29 (J = 2.13, 10.07Hz) and $\delta$ = 5.25 (J = 2.44, 9.16Hz) were C-2-H and C-3-H. Two quartets, at $\delta$ = 5.15 and $\delta$ = 4.79 represent C-4-H and C-1-H, C-5-H respectively, and indicated that these positions were phosphorylated.

The eight ethyl groups of **(275)** were removed by a simple transetherification reaction. The tetrakisphosphate was dissolved in dry dichloromethane and bromotrimethylsilane was added dropwise under nitrogen and stirred at room temperature overnight. The trimethylsilyl moieties replaced the ethyl functions to give bromoethane as a by-product. The transesterification reaction was quantitative by $^{31}$P NMR for the deprotection of the octaethylphosphate because the four peaks at -1.53, -2.11, -2.18 and -2.49ppm disappeared and four new signals appeared at -12.4, -13.2, -15.1 and -17.3ppm, which indicated an upfield shift due to the trimethylsilyl groups attached to the phosphate. The eight silicon protective groups were then hydrolysed quantitatively by the addition of water to give DL-3,6-di-*O*-benzoyl Ins(1,2,4,5)$P_4$ **(276)** (Figure 71). A small sample was purified by ion exchange chromatography using a gradient of TEAB on Q-Sepharose Fast Flow to give the pure compound **(276)** in 81% yield. The four phosphorus signals had moved downfield to -0.22 (d, J = 9.76Hz), -0.39 (d, J = 10.68Hz), -0.49 (d, J = 11.9Hz) and -0.79 (d, J = 8.83Hz).

DL-Ins(1,2,4,5)$P_4$ **(152)** was prepared by hydrolysis of the two benzoate esters of compound **(276)** with 1M aqueous sodium hydroxide at 60°C for 1h. An elevated temperature was used because the ester groups appeared to be resistant to basic hydrolysis at room temperature. After 1h the pH was adjusted to ~ 6 with Dowex ($H^+$ form) which was then filtered off and the benzoic acid was removed by washing the aqueous solution with dichloromethane. Pure DL-Ins(1,2,4,5)$P_4$ **(152)** was obtained after ion exchange chromatography, which eluted at *ca.* 550mM TEAB buffer.

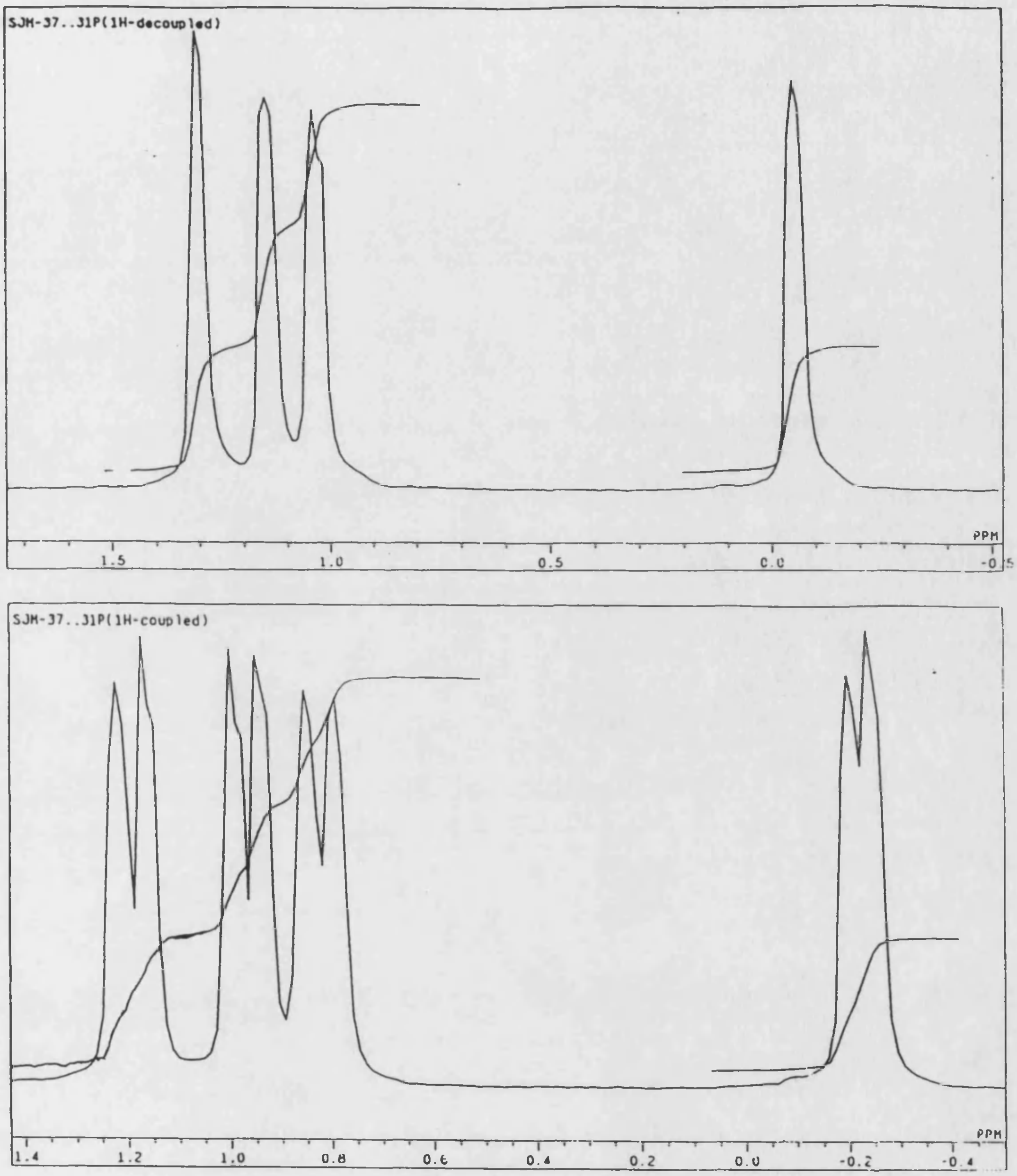



**Figure 73**

The $^{31}P$ NMR (Figure 73) shows four singlets in the $^{31}P$-$^{1}H$-decoupled spectrum and four doublets for the $^{31}P$ NMR $^{1}H$-coupled spectrum, +1.31 (d, J = 8.00Hz), +1.15 (d, J = 7.90Hz), +1.04 (d, J = 9.9Hz) and -0.04 (d, J = 6.0Hz). In the $^{1}H$ NMR spectrum, C-2-H was obscured by the HDO peak at δ = 4.80, C-4-H was a quartet at δ = 4.29 (J = 9.16Hz) but C-1-H and C-5-H overlapped at δ = 3.97-4.04 and both were phosphorylated. Positions C-3-H and C-6-H were not phosphorylated and were observed as a doublet, (J = 10.07Hz) and a triplet, (J = 9.46Hz) respectively.

### 5.2.1 Pharmacology

DL-3,6-Di-*O*-benzoyl Ins(1,2,4,5)$P_4$ **(276)** and DL-Ins(1,2,4,5)$P_4$ **(152)** have been examined for their ability to displace [$^3$H]Ins(1,4,5)$P_3$ from membranes prepared from bovine adrenal cortexes. [472] Compound **(152)** (Figure 74) displaced [$^3$H]Ins(1,4,5)$P_3$ (15-30Ci/mM, NEN), with a $K_i$ value of 26.4nM ± 1.8 whereas compound **(276)** did not compete (>3μM). As expected, **(276)** was devoid of $Ca^{2+}$-mobilising activity from $^{45}Ca^{2+}$-preloaded SH-SY5Y human neuroblastoma cells permeabilised with saponin (>100μM). However, it was found (by R. A. Wilcox) [472] that (**152** in Figure 75) released $^{45}Ca^{2+}$ potently from intracellular stores with an $EC_{50}$ value of 165nM, compared to an $EC_{50}$ value of 52nM for Ins(1,4,5)$P_3$.

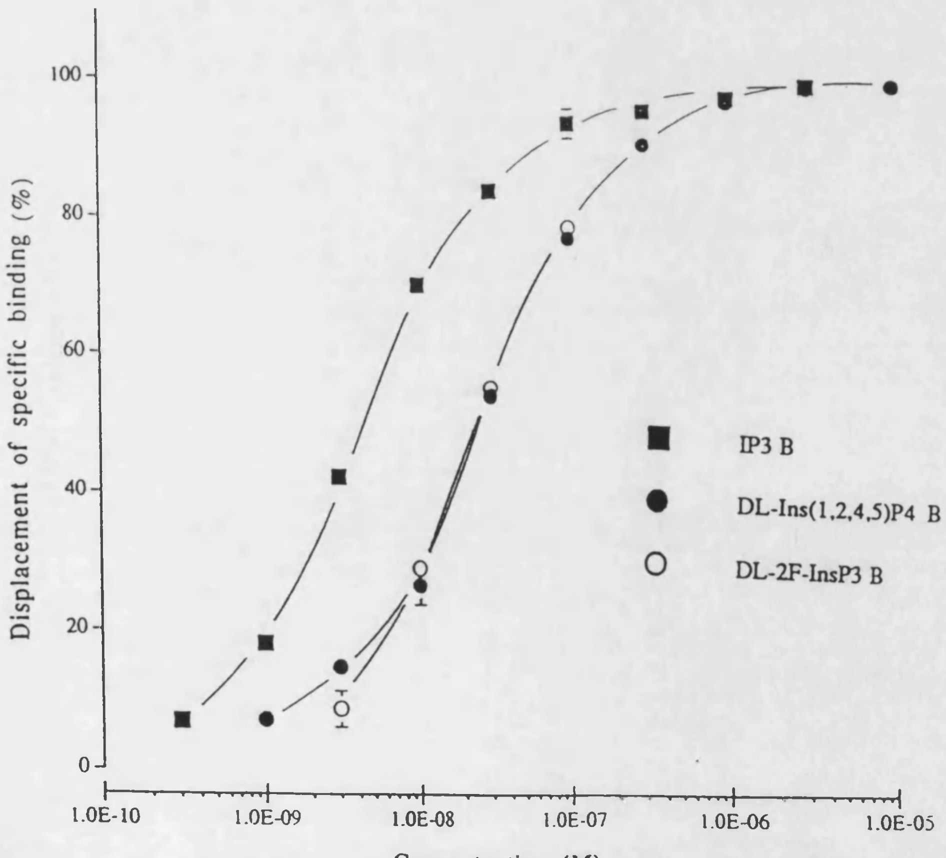



**Figure 74**

Both compounds were tested for their ability to interact with the enzymes 5-phosphatase from HEG and 3-kinase from CRBHS. Compound **(276)** had a low affinity for 3-kinase, with a $K_i$ value of 105μM [*cf.* $K_m$ for Ins(1,4,5)$P_3$ = 1.5μM], and inhibited the dephosphorylation of [$^3$H]Ins(1,4,5)$P_3$ with a $K_i$ value of 15.9μM [*cf.* $K_m$ for Ins(1,4,5)$P_3$ = 31.8μM]. Compound **(152)** did not inhibit [$^3$H]Ins(1,4,5)$P_3$ phosphorylation (>300μM), but was a potent inhibitor of Ins(1,4,5)$P_3$ 5-phosphatase, with a $K_i$ value of 2.9μM, under conditions where 10μM of Ins(1,4,5)$P_3$ (2.5nM) liberated 1.4nmol ± 0.5 of inorganic phosphate monitored colorimetrically as described previously. [473] Ins(1,4,5)$P_3$ 5-phosphatase did not liberate inorganic phosphate when incubated with either compound (10μM, 2.5nM). However, initial experiments (carried out by R. A. Wilcox) [472] monitoring the temporal aspects of $Ca^{2+}$-release from saponin-permeabilised SH-SY5Y neuroblastoma cells with the $Ca^{2+}$ sensitive dye, Fluo-3 (1μM) indicated the rate of reuptake of $Ca^{2+}$ was slightly slower than for Ins(1,4,5)$P_3$, suggesting that DL-Ins(1,2,4,5)$P_4$ was metabolised by an enzyme or enzymes other than 3-kinase or 5-phosphatase.

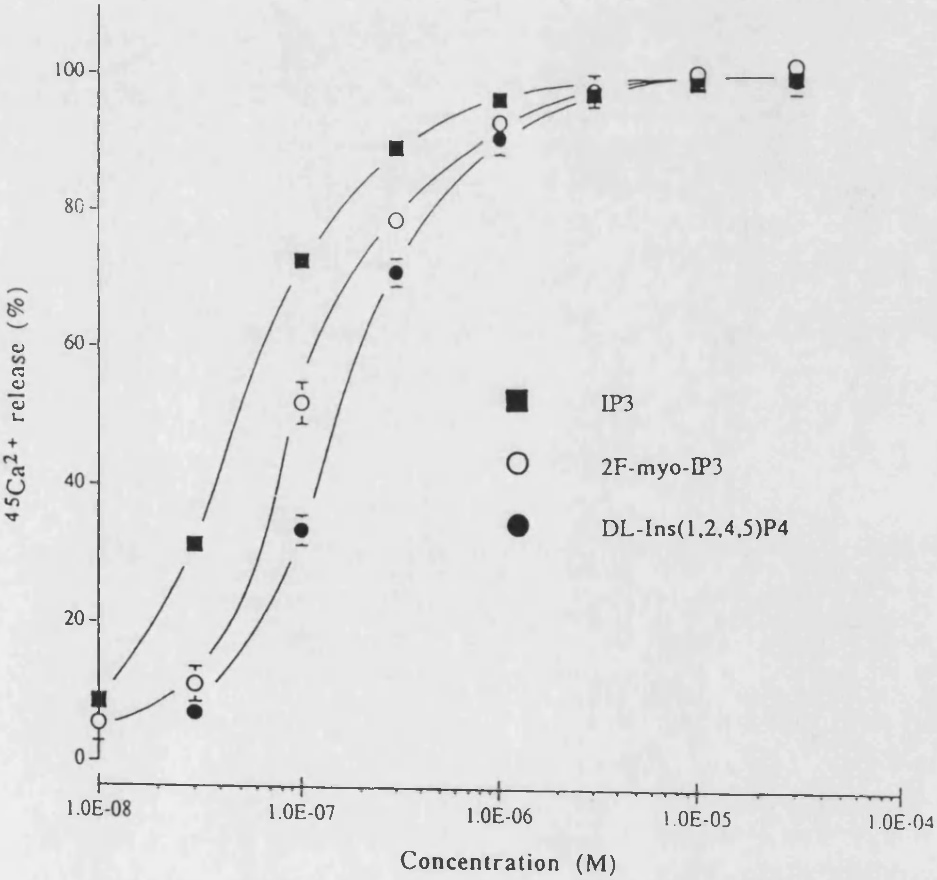



**Figure 75**

These results indicate that introduction of a bulky negatively charged phosphate group at the 2-position only slightly decreases $Ca^{2+}$-release from saponin-permeabilised SH-SY5Y neuroblastoma cells. However, the affinity for 5-phosphatase increased like other tetrakisphosphates such as Ins(1,3,4,5)$P_4$. However, the affinity decreased for 3-kinase in comparison to Ins(1,4,5)$P_3$. The benzoate groups of DL-3,6-di-*O*-benzoyl Ins(1,2,4,5)$P_4$ sterically interact with the enzyme 5-phosphatase, and decreased the binding affinity compared to DL-Ins(1,2,4,5)$P_4$ by approximately 6-fold.

### 5.3 The Synthesis of D- and L-Ins(1,2,4,5)$P_4$ and D-Ins(1,2,4,5)$PS_4$

#### 5.3.1 Synthesis of DL-1,4-Di-*O*-Benzyl-5,6-Di-*O*-*p*-Methoxybenzyl-*myo*-Inositol

The enantiomers of Ins(1,2,4,5)$P_4$ were synthesised by a different route to the racemic mixture. The reason for the synthesis of these two compounds was first, to establish which isomer was responsible for the inhibition of the enzyme 5-phosphatase and second, to discover the true $EC_{50}$ value for D-Ins(1,2,4,5)$P_4$ in comparison to Ins(1,4,5)$P_3$ and *scyllo*-Ins(1,2,4,5)$P_4$. It is also known that L-Ins(2,4,5)$P_3$ can release $Ca^{2+}$ from intracellular stores (see section 4.4.2) albeit with a low potency ($EC_{50}$ value of 110μM) and thus it would be interesting to discover if L-Ins(1,2,4,5)$P_4$ made some contribution to the $Ca^{2+}$-releasing properties observed for DL-Ins(1,2,4,5)$P_4$. *scyllo*-Ins(1,2,4,5)$PS_4$, described in section 4.9.3 was a partial agonist at the Ins(1,4,5)$P_3$ receptor and a single modification to give an axial phosphorothioate group produced D-Ins(1,2,4,5)$PS_4$ which may also be a partial agonist.

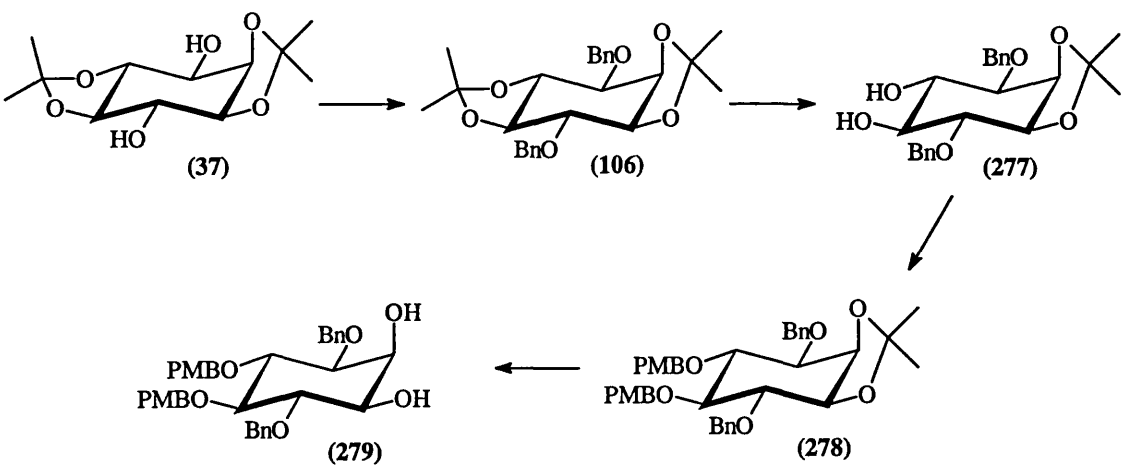



**Figure 76**

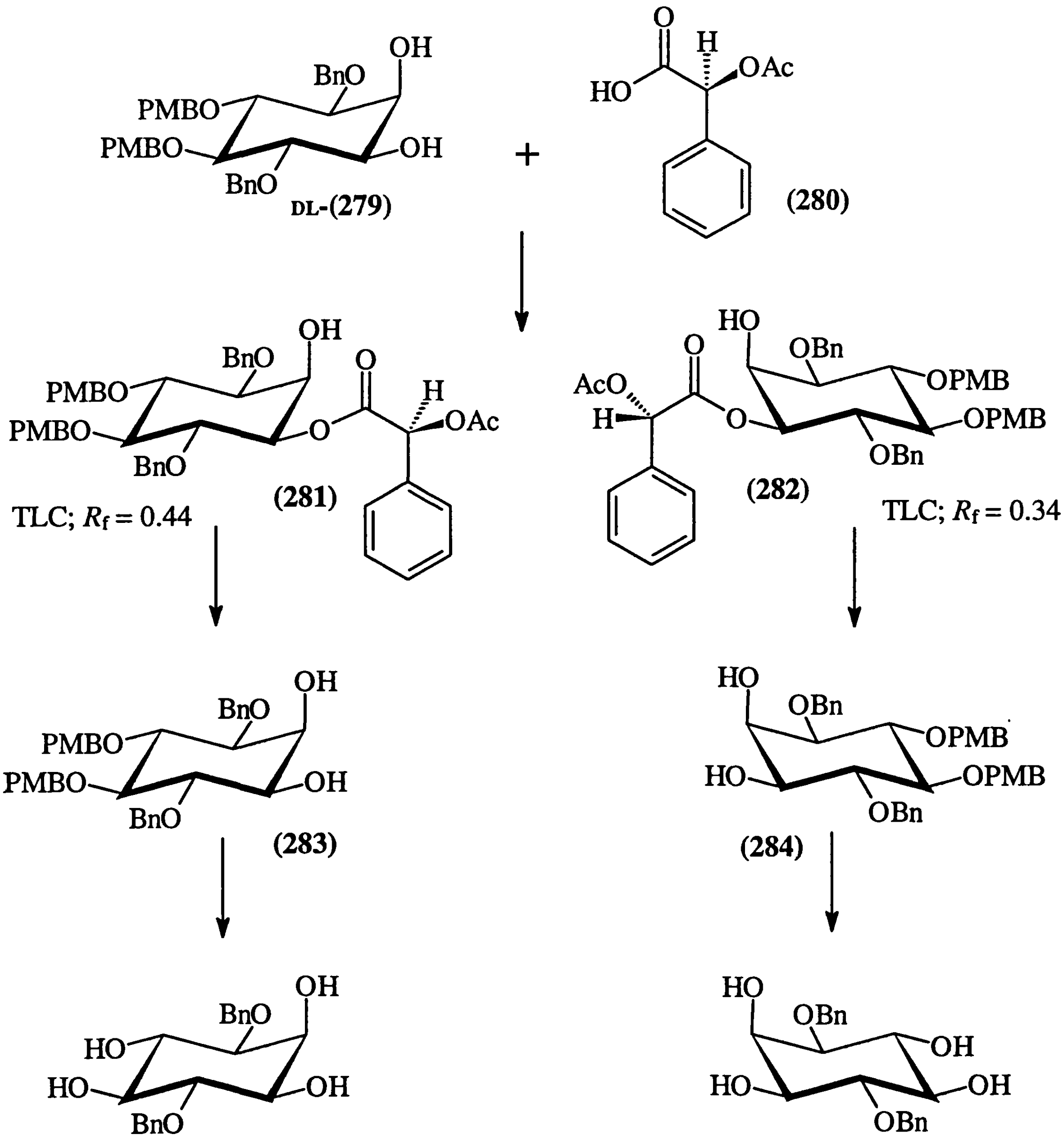
The racemic compound, DL-1,4-di-*O*-benzyl-5,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol (**279** in Figure 76) has been synthesised by another group using a different route. [474]

DL-3,6-Di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(36)** was prepared as described in section 5.2. Basic hydrolysis of the two benzoyl groups was followed by neutralisation with carbon dioxide and addition of water, which was then evaporated *in vacuo* to give the crude product. DL-1,2:4,5-Di-*O*-isopropylidene-*myo*-inositol **(37)** was extracted from the mixture with dichloromethane. It was found that if the crude solid contained moisture at this stage, a poor yield was obtained, and the extracted diol **(37)** was contaminated with benzoic acid. The crude mixture was then recrystallised from ethyl acetate to give the pure diol **(37)**. Benzylation of this intermediate with benzyl bromide and sodium hydride in DMF gave the highly crystalline DL-3,6-di-*O*-benzyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(106)**. The less stable *trans* acetal was removed selectively in 80% yield using a catalytic amount of toluene-*p*-sulphonic acid and ethane 1,2-diol (one equivalent) in dichloromethane at room temperature. The reaction was quenched with triethylamine at the instant the solution became cloudy due to the formation of DL-1,4-di-*O*-benzyl-*myo*-inositol. The mixture was worked up and purified by flash chromatography to provide DL-1,4-di-*O*-benzyl-2,3-*O*-isopropylidene-*myo*-inositol **(277)** in 80% yield and was the first stage in the route where chromatography was employed. This compound has also been prepared by Gigg and coworkers under different acidic conditions in 55% yield from compound **(106)**. A second, more efficient method for making this compound, required the synthesis of DL-1,2-*O*-isopropylidene-*myo*-inositol prepared from *myo*-inositol, 2,2-dimethoxypropane a catalytic amount of toluene-*p*-sulphonic acid in DMSO at 90°C, in 79% yield. DL-1,2-*O*-Isopropylidene-*myo*-inositol was then treated with two equivalents of dibutyltin oxide, tetrabutylammonium bromide in acetonitrile at reflux temperature. Work up and flash chromatography gave compound **(277)** in 62% yield. [475]

The hydroxyl groups at the 4- and 5-positions were alkylated with *p*-methoxybenzyl chloride in DMF with sodium hydride as base. Work up and purification by flash chromatography provided the pure compound **(278)** as a syrup. The $^1H$ NMR spectrum showed two singlets at $\delta$ = 3.77 and 3.79ppm for the -OMe group of the *p*-methoxybenzyl moieties. The *cis* 1,2-*O*-isopropylidene group was removed by careful acid treatment with 1M aqueous HCl in methanol (1:9) at 50°C for 30min, after which the *p*-methoxybenzyl groups were intact and the acetal had been removed to give compound **(279)**. Caution must be taken at this stage, because prolonged treatment of a compound containing a *p*-methoxybenzyl function with acid will result in its hydrolysis. With the presence of a *cis* 1,2-diol in compound **(279)** it was envisaged that introduction of a chiral acid at the equatorial 1-position would result in the formation of two diastereoisomers which may be separated by selective crystallisation or chromatography.

## 5.3.2 Optical Resolution of DL-1,4-Di-*O*-Benzyl-5,6-*O*-*p*-Methoxybenzyl-*myo*-Inositol

(*S*)-(+)-*O*-Acetylmandelic acid **(280)** was chosen for resolution of the *cis* 1,2-diol because it is relatively cheap (£23.70 for 5g) and is 99% pure by GLC (Aldrich 1994/95). Unlike the enantiomers of camphanic acid chloride (discussed in section 3.7) both (*R*)- and (*S*)-enantiomers are available at a similar price.




D-3,6-Di-*O*-benzyl **(285)** -*myo*-inositol

L-3,6-Di-*O*-benzyl **(286)** -*myo*-inositol

**Figure 77**

(*S*)-(+)-*O*-Acetylmandelic acid has not been widely used for the resolution of *myo*-inositol derivatives, so we took the opportunity to investigate its potential as a resolving agent. The resolutions in this thesis were successful, and the two diastereoisomers resulting from each racemic mixture were easily separated by flash chromatography.

The treatment of DL-1,4-di-*O*-benzyl-5,6-*O*-*p*-methoxybenzyl-*myo*-inositol **(279)** with (*S*)-(+)-*O*-acetylmandelic acid **(280)** (1.03 equivalents) in the presence of DMAP and 1,3-dicyclohexylcarbodiimide (DCC) at -20°C afforded two diastereoisomers **(281)** and **(282)** in 36% and 37% yield respectively (Figure 77). By keeping the temperature at -20 °C with dry ice, there was no acylation by NMR of the 2-position, thus selectivity was achieved and work up was easy. The simplicity of the resolution lies in the ease with which the two diastereoisomers were separated by flash chromatography. The two diastereoisomers **(281)** and **(282)** had $R_f$ values of 0.44 and 0.34 respectively (chloroform-acetone, 30:1). Both diastereoisomers were obtained as crystalline solids m.p. **(281)**: 120-121°C and **(282)**: 147-148°C and both had positive rotations $[\alpha]_D$ = +12° and $[\alpha]_D$ = +42° for **(281)** and **(282)** respectively.

The hydrogen at position one (shifted downfield due to esterification at the 1-hydroxyl position) could not be identified in either diastereoisomer (Figure 78) because the methylene AB coupling pattern of the benzyl group overlapped with the dd for C-1-H. However, C-2-H was identified as a broad doublet at $\delta$ = 4.15 (J = 1.83Hz) for compound **(281)** and at $\delta$ = 4.40 for compound **(282)** as a broad doublet (J = 1.83Hz). The C-2-OH signal was also significant because it was seen at $\delta$ = 2.16 for compound **(281)** and at $\delta$ = 2.69 for **(282)** which indicated that the hydrogen and hydroxyl groups at C-2 were more deshielded than for the less polar diastereoisomer **(281)**. The unique singlet at $\delta$ = 5.94 for **(281)** and at $\delta$ = 5.98 for **(282)** of $CH_3CO_2\mathit{CH}(Ph)CO_2$- is indicative of the high purity of the diastereoisomers.

Base catalysed deacylation of the two diastereoisomers gave pure enantiomers **(283)** and **(284)** where $[\alpha]_D$ = -25° and +25° respectively. Melting points were only slightly higher than for the racemic mixture, (m.p. 133-134°C for both enantiomers). The two chiral 1,2,4,5-tetrols were prepared by acid hydrolysis of the of the *p*-methoxybenzyl ethers in 1M HCl-ethanol (1:2) at reflux temperature for 4h. Deprotection of the *p*-methoxybenzyl groups with DDQ or CAN would have caused problems because the product is very polar and insoluble in solvents such as dichloromethane or ethyl acetate, thus work up and extraction of the product would have been difficult. After 4h the mixture was cooled and the solvents were evaporated *in vacuo*. The resulting solid was filtered and recrystallised from ethanol to give the pure enantiomers D-**(285)** and L-3,6-

di-*O*-benzyl-*myo*-inositol (**286**) which had specific rotations of +16° and -16° respectively. The melting point of the racemic mixture (lit. [344] 205-207°C) was much higher than for the chiral antipodes (172-173°C for both enantiomers).

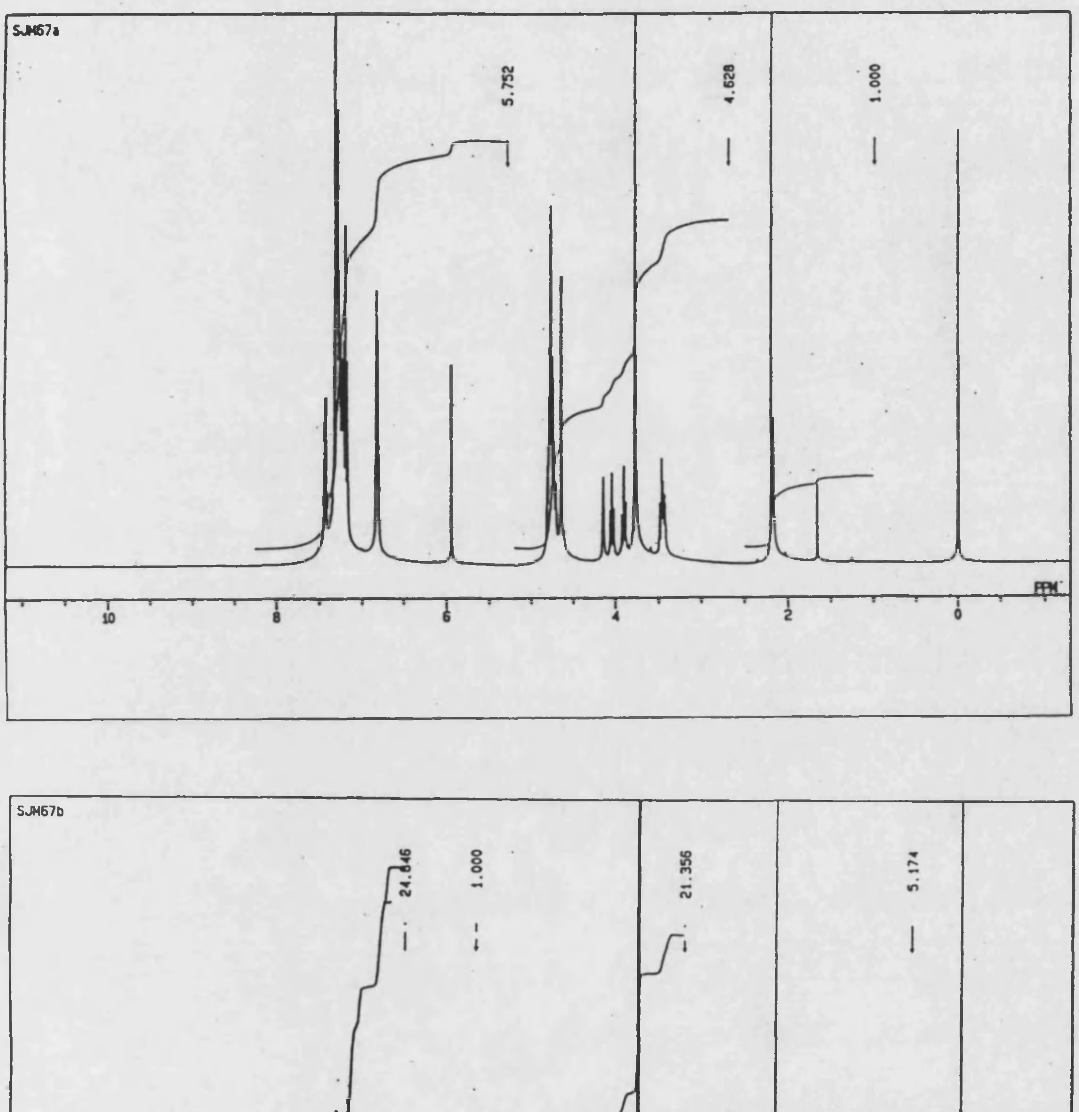



**Figure 78**

The $^{1}H$ NMR spectrum of compound **(285)** in $d_6$-DMSO is shown in Figure 79. The expansions below the full spectrum show the *myo*-inositol ring hydrogens, four of which are coupling to their respective hydroxyl groups, and two which are protected at positions 3- and 6-. Following $D_2O$ exchange, four of the ring hydrogen signals collapsed to give a triplet at $\delta = 3.97$ (C-2-H), a triplet at $\delta = 3.58$ (C-4-H), a dd at ($\delta =$ 3.31) C-3-H, and a triplet at $\delta = 3.13$ (C-5-H). This tetrol was the precursor for the synthesis of D-Ins(1,2,4,5)$P_4$ and the enantiomer from the more polar diastereoisomer **(282)**, L-3,6-di-*O*-benzyl-*myo*-inositol **(286)**, was the precursor of L-Ins(1,2,4,5)$P_4$.

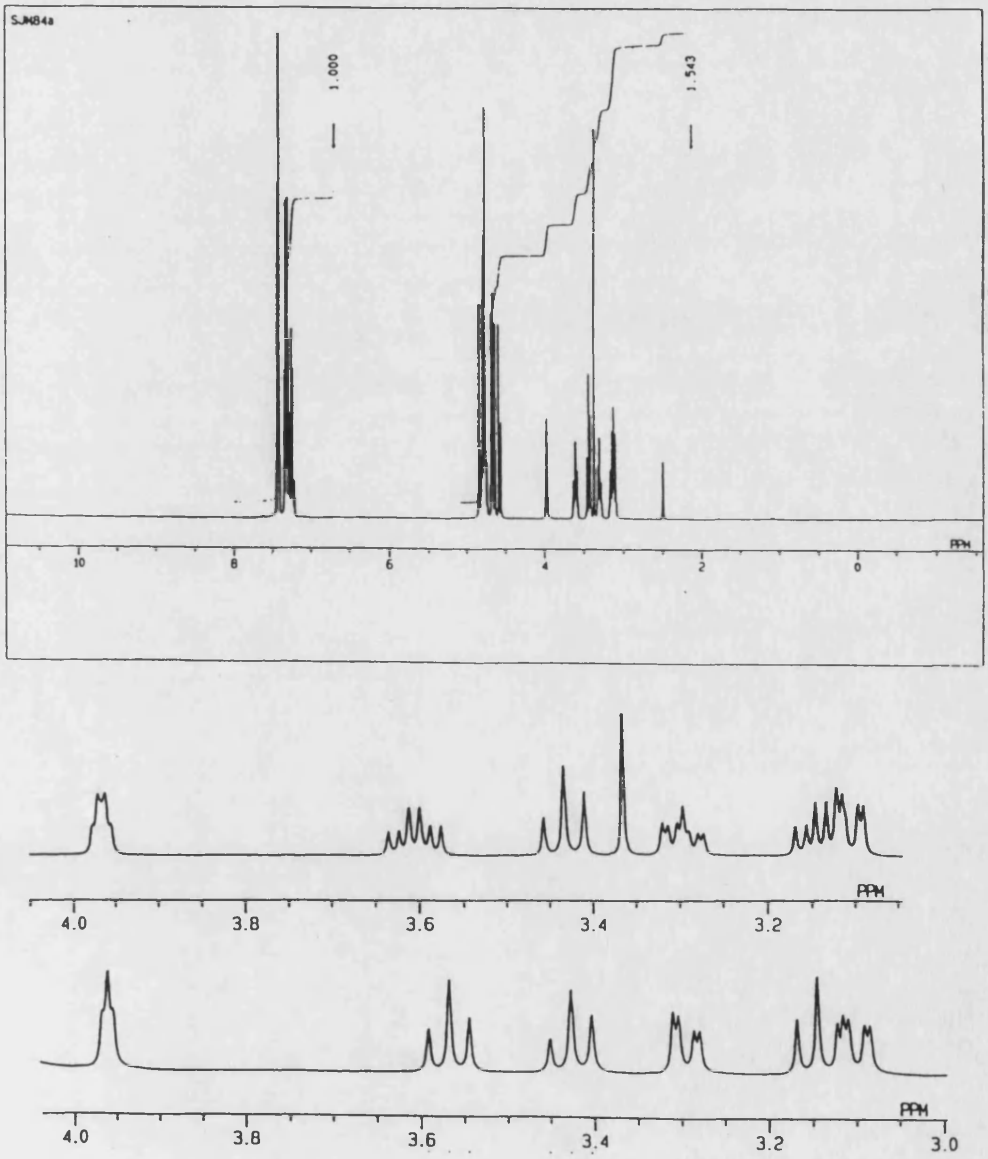
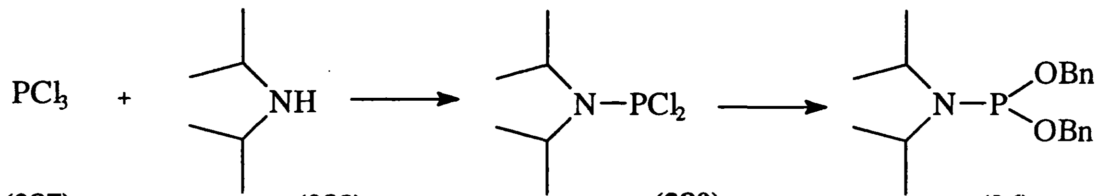



**Figure 79**

### 5.3.3 Preparation of Bis(benzyloxy)diisopropylaminophosphine

The phosphitylating reagent for the synthesis of most of the *myo*-inositol phosphates and phosphorothioates was bis(benzyloxy)diisopropylaminophosphine (**96** in Figure 80). *N,N*-Diisopropylaminodichlorophosphine **(289)** was prepared by Tanaka and coworkers [476] by careful addition of two equivalents of *N,N*-diisopropylamine **(288)**, dropwise, to an ethereal solution of phosphorus trichloride **(287)** at -78°C. The product was isolated by filtering the hydrochloride salt under nitrogen, which was then washed with more dry ether and evaporated to give a viscous moisture-sensitive syrup. The product was purified by distillation under reduced pressure ($\delta_P$ = +169.4ppm) and was stored as a crystalline solid at -20°C.




**(287)** **(288)** **(289)** **(96)**

**Figure 80**

Benzyl alcohol (2 equivalents) was then added to a solution of *N,N*-diisopropyl aminodichlorophosphine in the presence of triethylamine and dichloromethane at -78°C and stirred for a further 2h at room temperature. The best result was obtained when more dichloromethane was added to the mixture so the reaction went to completion and an excess of benzyl alcohol was not present which would cause problems with the phosphitylation procedure. The product was then isolated by filtering the triethylammonium chloride salt, and the dichloromethane layer was washed with a saturated aqueous solution of sodium hydrogen carbonate. The organic layer was then dried over magnesium sulphate, filtered and evaporated to give a thick syrup in 96% yield $R_f$ = 0.78, (hexane-triethylamine, 10:1). Purification by chromatography was not necessary, because the $^1$H NMR spectrum showed a clean sample without contamination from benzyl alcohol or triethylamine and the $^{31}$P-$^1$H-decoupled NMR spectrum showed a single peak at $\delta_P$ = +147.86ppm. The purity of the reagent **(96)** was evident from the phosphitylation procedure.

### 5.3.4 Preparation of D-Ins(1,2,4,5)$P_4$, L-Ins(1,2,4,5)$P_4$ and D-Ins(1,2,4,5)$PS_4$

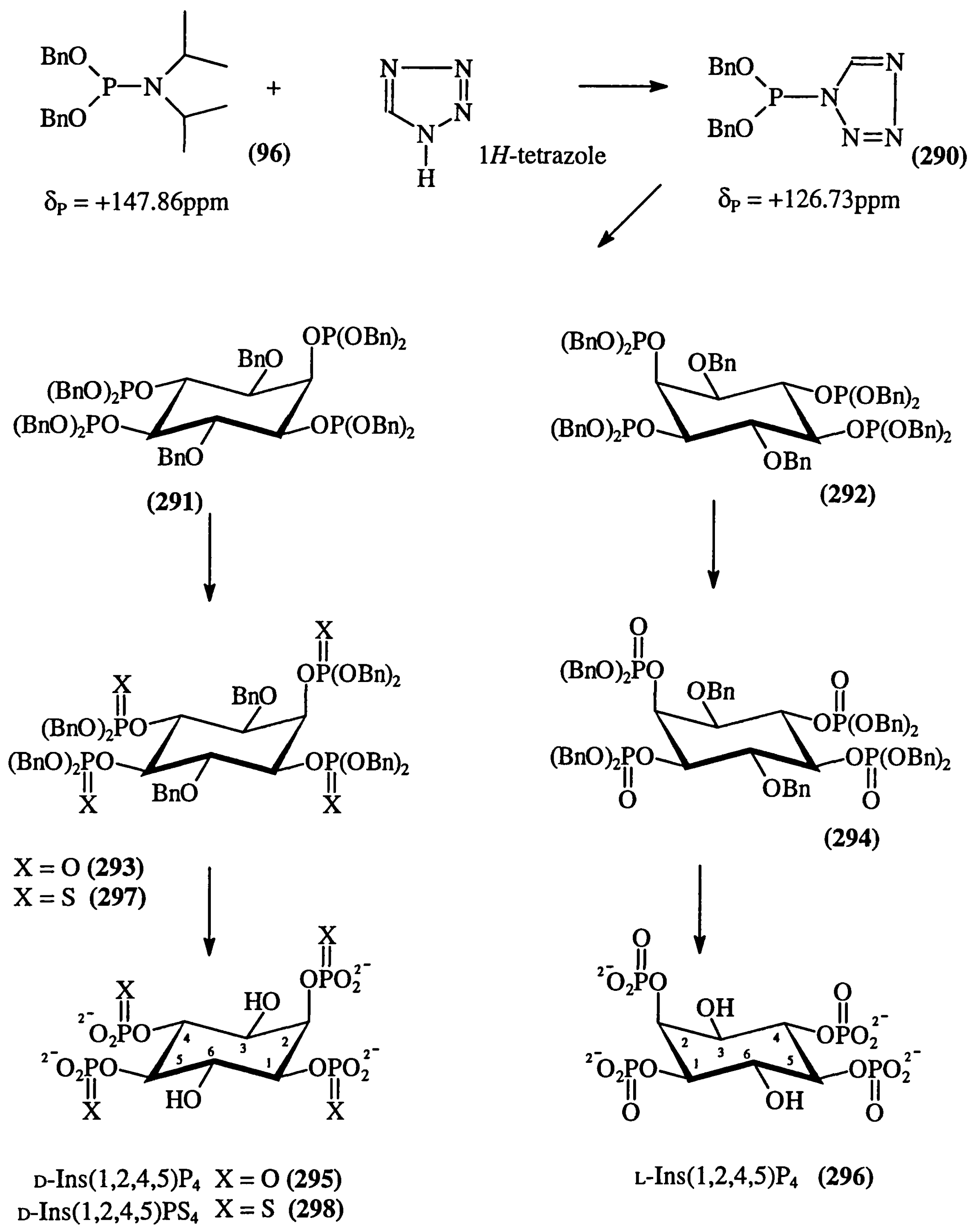



**Figure 81**

Phosphitylation of two pairs of vicinal diols (an axial-equatorial pair and an equatorial-equatorial pair) would be very difficult with some P(V) reagents due to their low reactivity and formation of undesirable cyclic phosphates. The most efficient method for introducing phosphates and phosphorothioates into the *myo*-inositol ring was to stir a mixture of phosphitylating reagent **(96)** (2 equivalents per hydroxyl) and 1*H*-tetrazole (4 equivalents per hydroxyl) in dry dichloromethane to form the tetrazolide intermediate (**290** in Figure 81). The $^{31}$P NMR at this stage showed a single peak at $\delta$ = +126.73ppm for **(290)** and no signal at $\delta$ = +147.86ppm for the phosphitylating reagent. Any moisture present in the solvent would be indicated by the presence of *H*-phosphonate at $\delta$ = +7.54ppm. For a successful phosphitylation procedure very little phosphonate should be present.

The procedure was carried out for both enantiomers in the same way. Thus, in separate experiments the enantiomers of 3,6-di-*O*-benzyl-*myo*-inositol **(285,286)** were added to the tetrazolide **(290)** in dichloromethane and stirred for a further 5min at room temperature. The $^{31}$P NMR spectrum again showed eight peaks and the distinctive five bond phosphorus-phosphorus spin-spin coupling systems for **(291,292)** ($^5J_{1,2}$ = 1.83Hz; $^5J_{4,5}$ = 3.66Hz) and is shown in Figure 72. Oxidation of the tetrakisphosphite intermediate with *m*CPBA at 0°C destroyed the two AB coupling patterns and provided the fully protected D-**(293)** and L-3,6-di-*O*-benzyl-1,2,4,5-tetrakis(dibenzyloxyphospho)-*myo*-inositol **(294)** in 94% and 88% yields respectively, after work up and purification by flash chromatography. The chosen solvent systems were important because chloroform-acetone (5:1) removed the phosphonate impurities and the pure enantiomers were eluted with ethyl acetate-pentane (2:1). The use of *m*CPBA superseded *t*-butylhydroperoxide because of lower yields resulting from the formation of other by-products present on the baseline. The problem of low yields and other by-products has been highlighted by Yu and Fraser-Reid. [375] The specific rotations for **(293)** and **(294)** were $[\alpha]_D$ = -3.5° (c = 2 in $CH_2Cl_2$) and $[\alpha]_D$ = +3.3° (c = 1.26 in $CH_2Cl_2$) respectively.

A deprotection strategy was employed to remove all the benzyl groups in one step with freshly cut sodium in distilled liquid ammonia and in the absence of a protic solvent. Under these conditions, the solution of sodium in liquid ammonia remained a deep blue colour due to electron solvation of the sodium. Dioxan was used to dissolve the compound because at -33°C it solidified, thus fixing the compound and holding it in solution. Unsatisfactory results have been obtained using THF as the solvent. The chemical reduction was complete within 2min, after which methanol was added to quench the reaction. The ammonia was evaporated under a stream of nitrogen and the crude mixture was dissolved in water. As a result of a clean deprotection there was a distinctive smell of 1,2-diphenylethane, generated as a by-product and which has a woody odour.

Purification of crude D-Ins(1,2,4,5)$P_4$ **(295)** and L-Ins(1,2,4,5)$P_4$ **(296)** was carried out by ion exchange chromatography on Q-Sepharose Fast Flow. Both compounds eluted at *ca.* 700 mM TEAB buffer and were isolated in 40% and 22% yields respectively. The proton and phosphorus NMR spectra for L-Ins(1,2,4,5)$P_4$ are given in Figure 82. The $^{31}P$ NMR spectrum showed four distinct signals at $\delta = +1.78$ (d, J 10.1Hz), $\delta = +1.44$ (d, J 6.7Hz), $\delta = +1.20$ (d, J 6.7Hz) and $\delta = +0.67$ (d, J 6.7Hz). The $^{1}H$ NMR showed five peaks accounting for six protons, $\delta = 4.71$ (1H, d, obscured by HDO peak) for C-2-H, $\delta = 4.30$ (1H, q, J 9.52Hz) for C-4-H, 4.03 (2H, q, J 9.34Hz) for C-1-H and C-5-H, 3.90 (1H, t, J 9.53Hz) and 3.72 (1H, d, J 9.71Hz), the extra peak at $\delta = 3.66$ is probably an amine impurity from the triethylamine. The specific rotations using TEAB as a solvent were $[\alpha]_D = -27.2°$ for **(295)** and $[\alpha]_D = +25.8°$ for **(296)**.

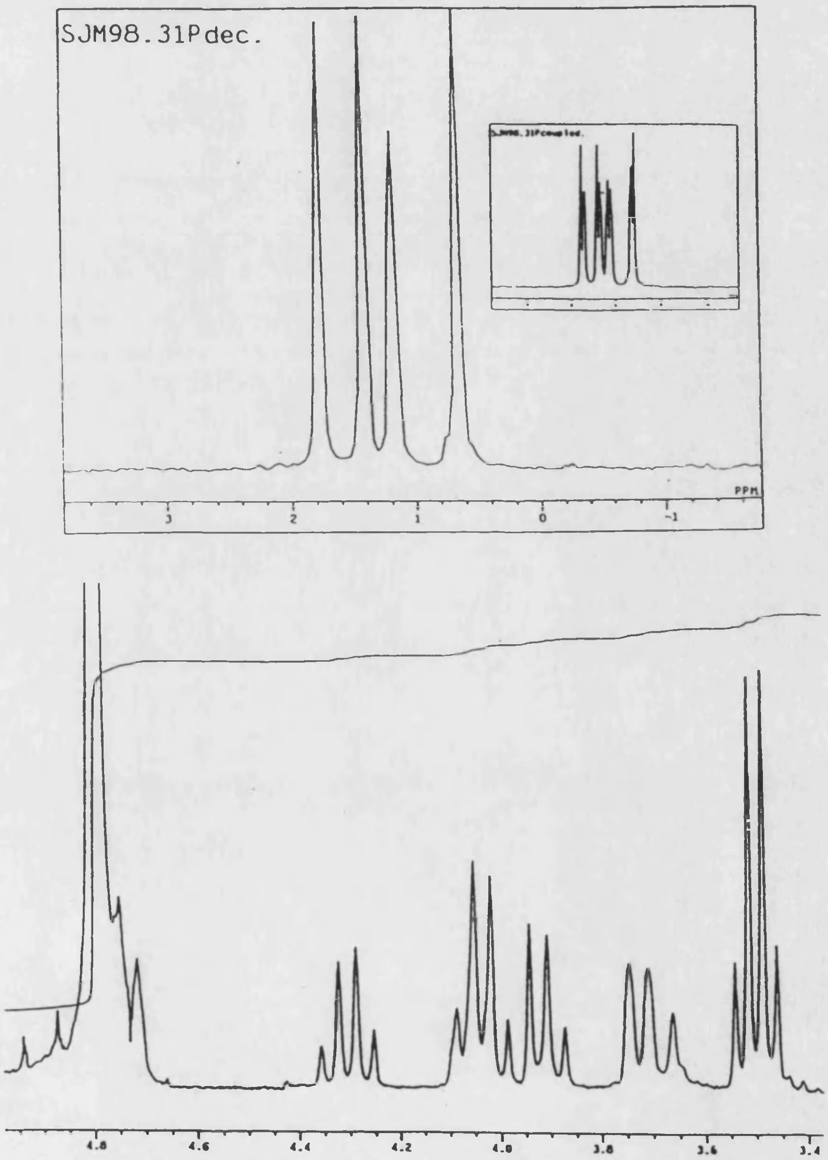



**Figure 82**

D-*myo*-Inositol 1,2,4,5-tetrakisphosphorothioate **(298)** was prepared *via* the tetrakisphosphite which was oxidised with sulphur to give the tetrakisphosphorothioate. The usual method for sulphoxidation contained a mixture of an excess of sulphur suspended in pyridine with the phosphite. The reaction is usually stirred overnight, followed by work up and chromatography. At this stage, the excess sulphur is difficult to remove completely. However, a fast sulphoxidation procedure (within 5min) was developed which provides clean fully blocked phosphorothioate compounds without such contamination with sulphur. At the tetrakisphosphite stage, the dichloromethane was evaporated to give a thick syrup. A mixture of dry DMF (2ml) and dry pyridine (1ml) was added together with sulphur (2 equivalents per phosphite). Sulphoxidation was complete within 5min, the excess sulphur was removed by filtration through cotton wool in a Pasteur pipette and the solvents were evaporated *in vacuo* at room temperature. Caution must be taken at this stage, because desulphurisation occurred when evaporation of the solvent was carried out at 70°C to give the symmetrical tetrabenzyl thiopyrophosphate at ($\delta$ = +56ppm), but no problem was encountered if evaporation was carried out at room temperature. The resulting syrup was diluted with ethyl acetate, washed with brine, dried and purified by flash chromatography (ether-pentane, 1:2) to give the fully blocked D-3,6-di-*O*-benzyl-1,2,4,5-tetrakis[di(benzyloxyphosphorothio)]-*myo*-inositol **(297)** as a clean syrup, having a specific rotation of -5.3° (c = 1.9 in $CH_2Cl_2$). Deprotection of **(297)** with sodium in liquid ammonia and final purification by ion exchange chromatography on Q-Sepharose Fast Flow eluted at *ca.* 700mM TEAB to provide D-Ins(1,2,4,5)$PS_4$ in 33.2% yield. The specific rotation was similar to that of D-Ins(1,2,4,5)$P_4$ where $[\alpha]_D$ = -25.8° (c = 0.31 in TEAB, at pH = 8.6). The $^1$H NMR of D-Ins(1,2,4,5)$PS_4$ showed five peaks similar to that of D-Ins(1,2,4,5)$P_4$ where $\delta$ = 4.83 (1H, d, J 9.46Hz) for C-2-H, $\delta$ = 4.46 (1H, q, J 11.92Hz) for C-4-H, 4.18 (2H, q, J 9.77Hz) for C-1-H and C-5-H, 3.86 (1H, t, J 9.46Hz) and 3.61 (1H, d, J 9.71Hz). The $^{31}$P NMR showed four signals at $\delta$ = 51.47 (d, J 12.18Hz), $\delta$ = 50.64 (d, J 10.26Hz), $\delta$ = 50.20 (d, J 11.54Hz) and $\delta$ = 49.14 (d, J 11.54Hz). The coupling constants for phosphorothioates coupling to a ring proton are usually larger than for the phosphate counterparts. The tetrakisphosphates and tetrakisphosphorothioate also gave satisfactory accurate -ve FAB spectra.

### 5.3.5 Pharmacology

At present these analogues have not been pharmacologically evaluated in terms of recognition by the Ins(1,4,5)$P_3$ receptor and the interaction with the enzymes 3-kinase and 5-phosphatase.

## 5.3.6 Establishing the Absolute Configuration of D-3,6-di-*O*-Benzyl-*myo*-Inositol

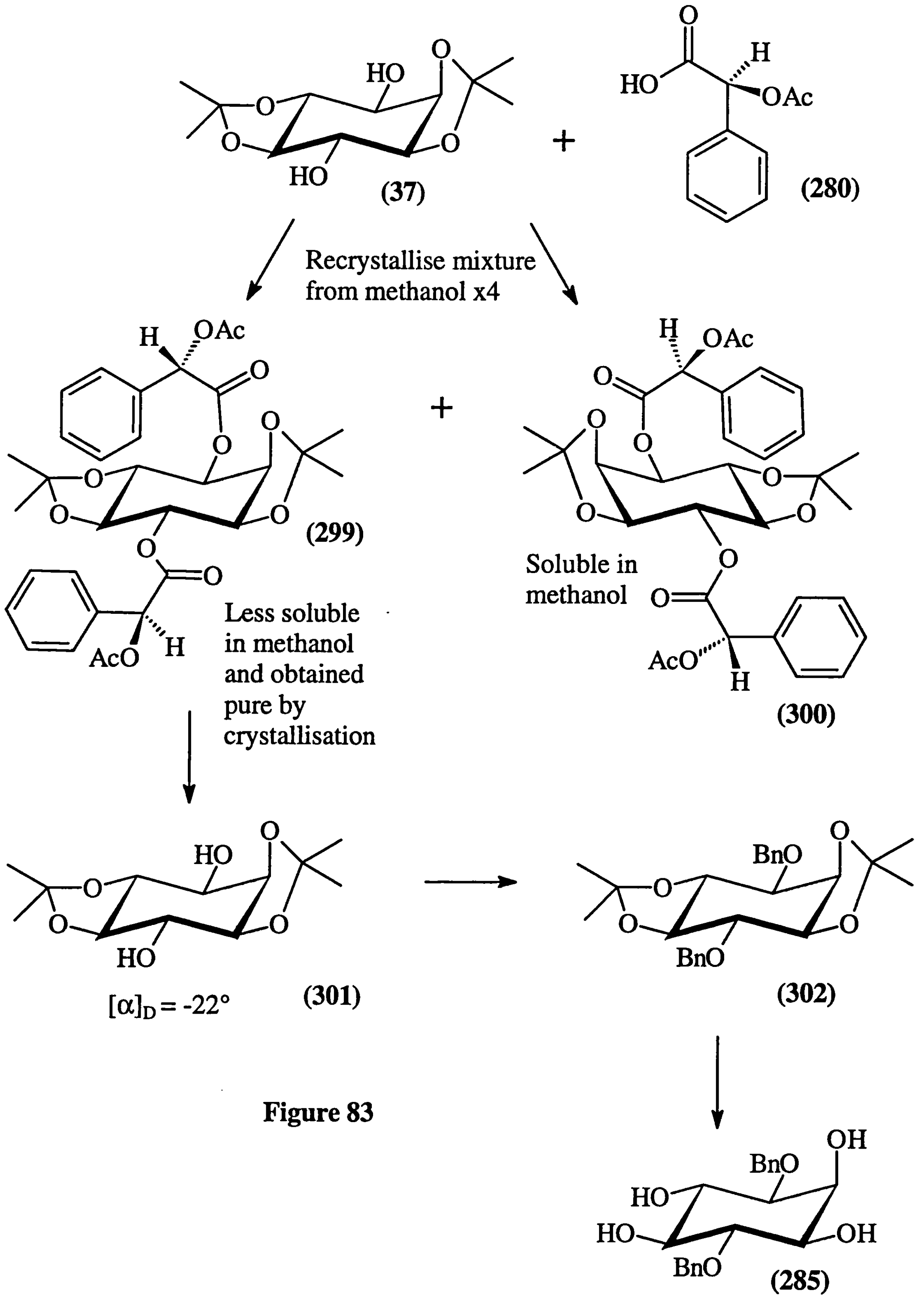



**Figure 83**

A simple way to establish the absolute configuration of D-3,6-di-*O*-benzyl-*myo*-inositol would be to resolve DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol followed by benzylation and acidic hydrolysis of the isopropylidene groups to give the individual enantiomers. The resolution of DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol has been previously accomplished using (*S*)-(-)-ω-camphanic acid chloride as the chiral auxillary. In this resolution, the 3-position was blocked using *t*-butyldiphenylsilyl chloride and the 6-position was acylated with the chiral auxillary and separation was carried out by tedious HPLC. Thus, a simple resolution to provide the chiral diol would be appropriate, because single X-ray crystal analysis of the 6-*O*-camphanate has been determined, and derived from this, the specific rotation, $[\alpha]_D$ = +22° and melting point (159-161°C) for L-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol has been established. [477] Moreover, in a recent article, in which D-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol was synthesised from D-mannitol, the melting point was found to be 176-177°C with $[\alpha]_D$ = -21.7°. [478] Thus, the specific rotation is in agreement for both enantiomers, but there appears to be some discrepancy over the melting point.

The isolation of the pure enantiomer D-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(301)** was carried out in the following manner and is outlined in Figure 83. DL-1,2:4,5-Di-*O*-isopropylidene-*myo*-inositol **(37)**, DCC and a catalytic amount of DMAP were stirred in dry dichloromethane at 0°C. (*S*)-(+)-*O*-Acetylmandelic acid **(280)** (1.25 equivalents per hydroxyl) in dichloromethane was added dropwise and the mixture was stirred at room temperature for a further 24h. The mixture was filtered through celite to remove the DCU and it was found TLC (including chloroform-acetone mixtures) did not separate the two diastereoisomers. The mixture of diastereoisomers was dissolved in hot methanol and allowed to cool. Figure 84 shows the mixture of diastereoisomers after one recrystallisation. One compound was in abundance by a factor of 2.5. Three further recrystallisations from the same solvent gave the pure diastereoisomer shown in Figure 85. The $^1$H NMR of the mixture of diastereoisomers in Figure 84 demonstrated several important features. First, the $^1$H NMR of the minor compound showed a methyl singlet (from the isopropylidene acetal) at δ = 1.00. Usually, the range for the methyl resonance for this type of functional group is δ = 1.30-1.65. In the mixed spectrum the two C-5-H resonances were identified at δ = 3.22 and 3.44 as two dd, for the major and minor compounds. Two other hydrogen resonances were also well separated at δ = 4.54 and 4.33 for C-2-H and at δ = 4.15 and 3.78 for C-1-H for the major and minor diastereoisomers respectively and for the pure compound (**299** in Figure 85) these minor peaks were not present. The unique peak to assess the purity of the diastereoisomer in Figure 85 was the hydrogen of the chiral acetylmandelates (at δ = 6.00 and 6.12) [$CH_3CO_2$*CH*(Ph)$CO_2$-]. The $^1$H NMR spectrum of the mixture (in Figure 84) showed a distinct singlet at δ = 6.05 which arose from the minor component and which was not present in the $^1$H NMR spectrum of **(299)** (Figure 85).

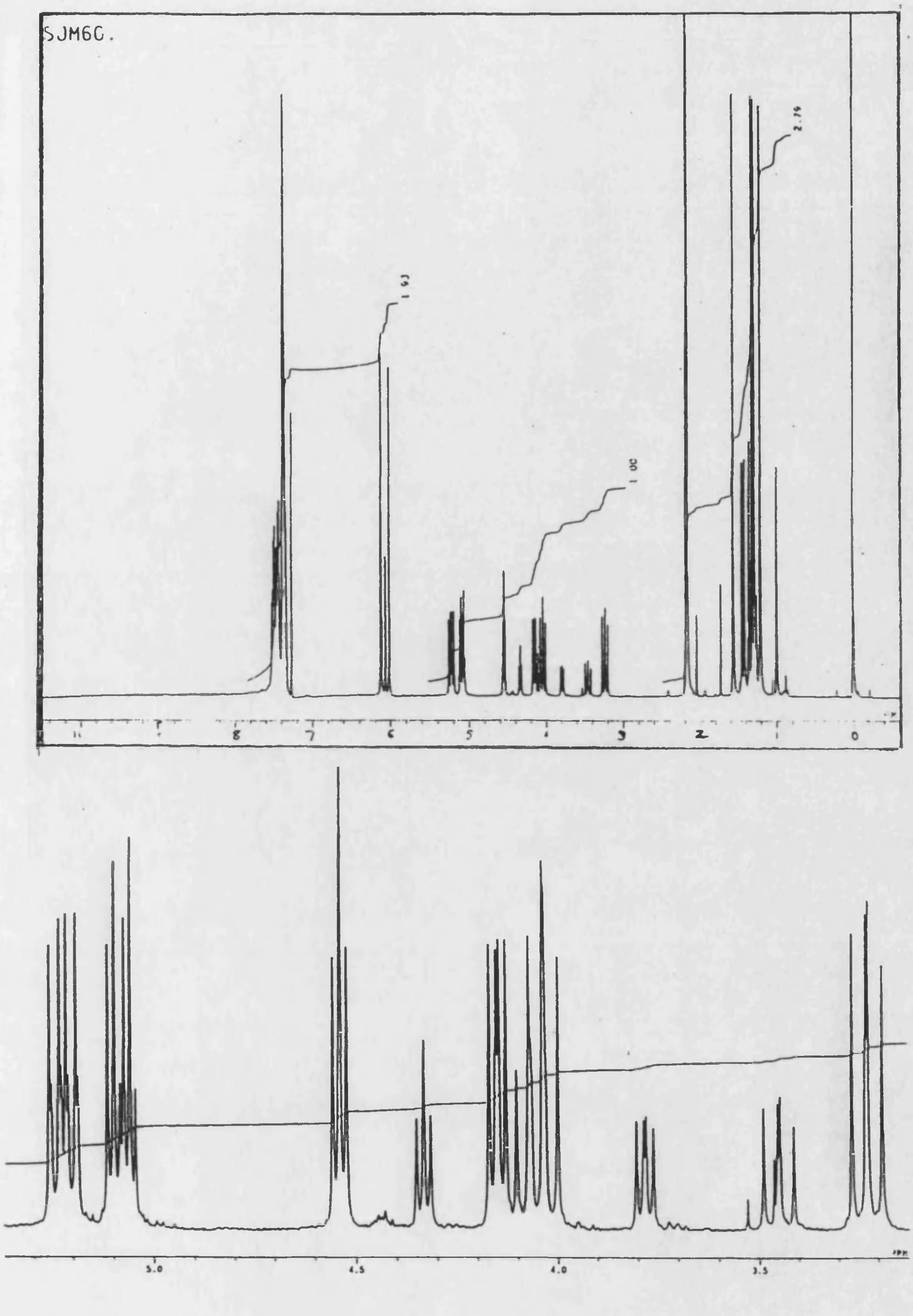



Figure 84

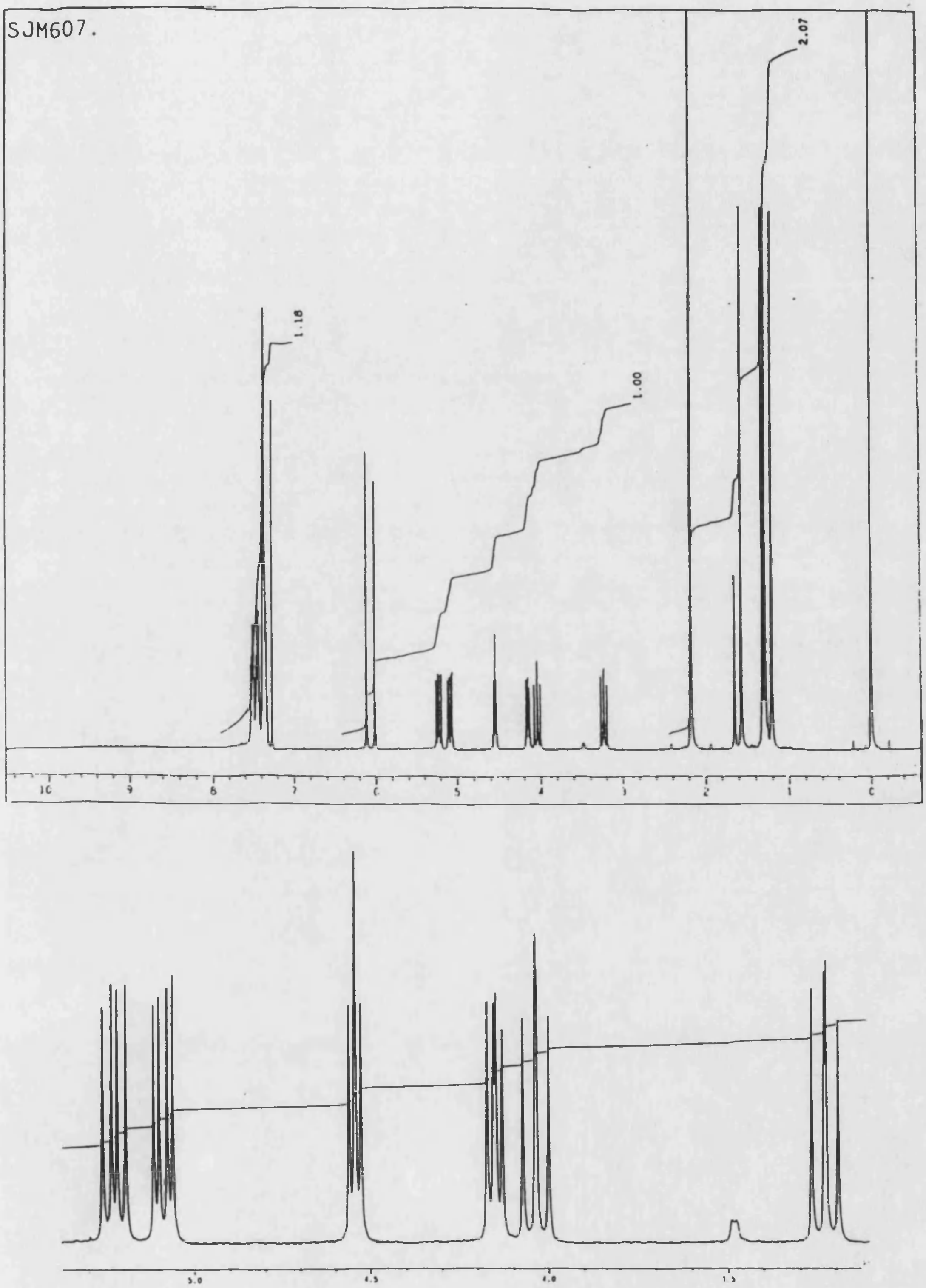



**Figure 85**

The yield was not particularly high (36.5%) for the recovery of a single diastereoisomer. However, this has not been optimised at present. The rotation was constant after a total of four recrystallisations, $[\alpha]_D = +64°$ together with the melting point (212-214°C). Basic hydrolysis of the two acyl groups followed by chromatography and recrystallisation of the diol from ethyl acetate gave D-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(301)** $[\alpha]_D = -22°$ with a melting point of 174-176°C. These physical properties agreed with the data published by Chiara and Martín-Lomas. [478] Gigg and coworkers [475] have synthesised L-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol by a different route, $[\alpha]_D = +23.3°$, (m.p. 175-177°C). The dispute over the melting point of the chiral diol has now been resolved because the value (159-161°C) stated by Young and coworkers [477] was incorrect. The hydroxyl groups at C-3-H and C-6-H were then benzylated to give D-3,6-di-*O*-benzyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(302)**, ($[\alpha]_D = -44°$, m.p. 157-159°C). Recently, Gigg and coworkers [475] synthesised L-3,6-di-*O*-benzyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol, $[\alpha]_D = +85°$, which had a melting point of 159-161°C. The acetal protecting groups at the 1,2- and 4,5-positions were removed by acid hydrolysis and the solvents were evaporated *in vacuo*. The resulting solid was recrystallised from ethanol to give D-3,6-di-*O*-benzyl-*myo*-inositol **(285)**, which was identical to the $^1$H NMR and had the same melting point and specific rotation described previously for **(285)** in section 5.3.4.

### 5.4 Modification of the Ins(1,3,4,6)$P_4$ Structure

Ins(1,3,4,6)$P_4$ is a partial agonist at the Ins(1,4,5)$P_3$ receptor from SH-SY5Y human neuroblastoma cells. Modifying the Ins(1,3,4,6)$P_4$ structure, may lead to the development of a full antagonist. In other systems however, for example in platelets, this tetrakisphosphate was shown to be a full agonist, thus receptor selectivity has also been achieved. Acting on this important finding, we decided to explore the roles of the hydroxyl groups and phosphate moieties of the *myo*-inositol ring. First, if the 2- and 5-positions of Ins(1,3,4,6)$P_4$ were methylated any polar interaction between the hydroxyl groups and the Ins(1,4,5)$P_3$ receptor protein would be removed, introducing steric hindrance, and this would demonstrate the importance of one or both of the hydroxyl groups at these positions. Second, replacing the phosphate groups with phosphorothioates at all four positions may provide a partial agonist with a lower efficacy than Ins(1,3,4,6)$P_4$, for reasons discussed in 4.9.3.

### 5.4.1 Synthesis of 2,5-Di-*O*-Methyl-*myo*-Inositol and 2,5-Di-*O*-Benzyl-*myo*-Inositol

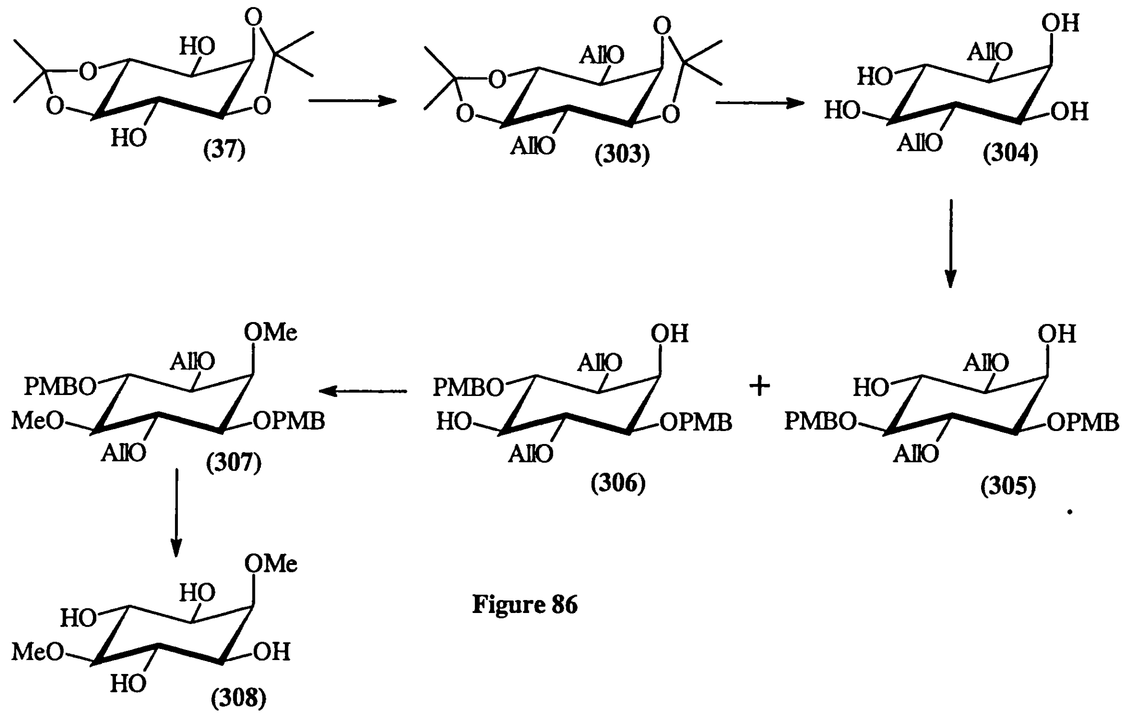


**Figure 86**

The two *meso* tetrols were synthesised from the same crystalline intermediate, DL-1,4-di-*O*-allyl-3,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol (**306** in Figure 86) which was prepared in three steps from DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol. The diol was alkylated with allyl bromide using sodium hydride as base in DMF. The reaction proceeded smoothly to give a single product by TLC, ether ($R_f$ = 0.8). The literature [349] stated that the pure compound was crystallised from the mixture. However, it was found that a pure compound was only isolated if there was little allyl bromide left after the reaction was complete. Crystallisation of DL-3,6-di-*O*-allyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(303)** occurred only at -20°C in the freezer because the product was highly soluble in the crystallising solvent (*i.e.* pentane) at room temperature. DL-1,4-Di-*O*-allyl-*myo*-inositol **(304)** was prepared by treating the fully protected derivative **(303)** with 80% acetic acid at reflux temperature for 30min. The acid was evaporated *in vacuo* to give a white solid which was then dissolved in hot ethanol, cooled and kept in the freezer until crystals appeared. It was envisaged that selective *p*-methoxybenzylation at the 3- and 6-positions would give a protected 1,3,4,6-intermediate which could be alkylated at the 2- and 5-positions to provide the 1,3,4,6-tetrol precursor. Thus, selective *p*-methoxybenzylation occurred by refluxing dibutyltin oxide (2.5 equivalents) with DL-1,4-di-*O*-allyl-*myo*-inositol **(304)** in toluene under reflux to form the *cis*-1,2 and the *trans*-4,5-dibutylstannylene derivative. The toluene was evaporated and DMF was added to the syrup together with CsF (5 equivalents) and *p*-

methoxybenzyl chloride (3 equivalents) and the reaction was then stirred overnight. The role of the CsF in this type of selective reaction has been discussed in Chapter 3. TLC (ethyl acetate-hexane, 1:1) showed two products, DL-1,4-di-*O*-allyl-3,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol **(306)** (isolated in 24% yield) ($R_f$ = 0.56) and DL-1,4-di-*O*-allyl-2,5-di-*O*-*p*-methoxybenzyl-*myo*-inositol **(305)** (isolated in 12% yield) ($R_f$ = 0.40). It was impossible to judge by TLC whether or not most of the dibutyltin acetal derivative collapsed to reform DL-1,4-di-*O*-allyl-*myo*-inositol, because of the overlapping $R_f$ values of the decomposed tin residues and the tetrol. This procedure was repeated several times and gave the same result. The protected derivatives DL-1,3,4,5-tetra-*O*-allyl-*myo*-inositol and 1,3,4,6-tetra-*O*-allyl-*myo*-inositol have also been synthesised in one step from *myo*-inositol and discussed in Chapter 3. However, both these compounds were syrups and not as easy to handle as compounds **(305)** and **(306)** which were crystalline solids. The diol **(306)** was methylated with methyl iodide using sodium hydride as the base in DMF. DL-1,4-Di-*O*-allyl-3,6-di-*O*-*p*-methoxybenzyl-2,5-di-*O*-methyl-*myo*-inositol **(307)** was isolated in 90% yield as a crystalline solid, m.p. 93-94°C.

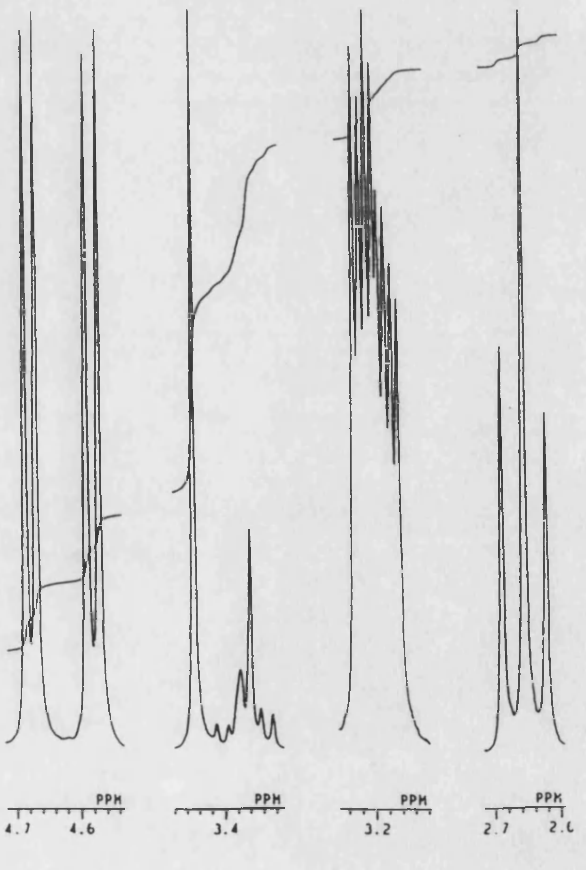
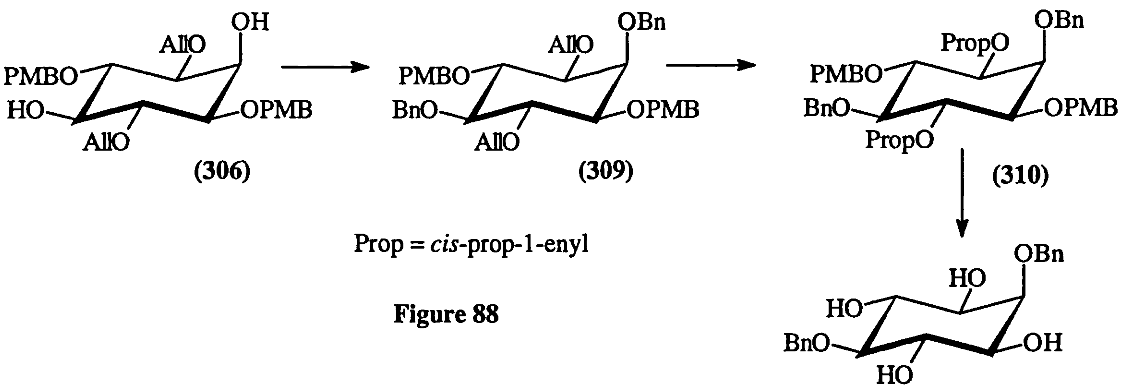



**Figure 87**

The allyl and *p*-methoxybenzyl protective groups were then removed in one step. The most efficient method for the deprotection was by refluxing a mixture of compound **(307)** in ethanol-water together with palladium on carbon and toluene-*p*-sulphonic acid overnight. The palladium on carbon was removed by filtering the solution through celite and the solvents were evaporated. Recrystallisation of the crude mixture from ethanol gave the *meso* compound 2,5-di-*O*-methyl-*myo*-inositol **(308)**. Whilst this work was being carried out, Gigg and coworkers [377] also prepared 2,5-di-*O*-methyl-*myo*-inositol **(308)** which had a melting point of 270°C, but we found that compound **(308)** decomposed between 266-268°C which was not observed by Gigg and coworkers. The $^1$H NMR of this compound in $d_6$-DMSO is shown in Figure 87. The $^1$H NMR spectrum indicated the presence of a symmetrical compound. There was a triplet at $\delta = 2.65$ for C-5-H (J 9.0Hz), a ddd, at $\delta = 3.19$, for C-1-H and C-3-H (J 2.56, 5.31, 9.89Hz), C-2-H was a broad singlet at $\delta = 3.35$ and there was a dt at $\delta = 3.36$ for C-4-H and C-6-H (J 4.95, 10.1Hz). Only two peaks were identified as doublets at $\delta = 4.57$ (J = 5.13Hz) and $\delta = 4.67$ (J = 4.76Hz), both hydroxyl groups exchanged with $D_2O$. A singlet at $\delta = 3.44$ was identified as the two methyl groups at the 2- and 5-positions. This intermediate was used for the synthesis of 2,5-di-*O*-methyl-*myo*-inositol-1,3,4,6-tetrakisphosphate and the phosphorothioate analogue.




(306) (309) (310) (311)

Prop = *cis*-prop-1-enyl

**Figure 88**

2,5-Di-*O*-benzyl-*myo*-inositol **(311** in Figure 88) was prepared in three steps from DL-1,4-di-*O*-allyl-3,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol **(306)**. Thus, **(306)** was benzylated using benzyl bromide and sodium hydride in DMF. Work up and purification gave the fully protected compound **(309)** as a highly crystalline solid in 92% yield. Several methods were attempted to deprotect the allyl and *p*-methoxybenzyl groups. First, palladium on charcoal at reflux temperature in an ethanol-water mixture and in the presence of toluene-*p*-sulphonic acid. Second, a two step deprotection, first with DDQ in (water-dichloromethane, 1:15), followed by deallylation with palladium on charcoal. Neither method was satisfactory for removing both protective groups.

However, a successful two step deprotection strategy was finally employed using freshly sublimed potassium *t*-butoxide in dry DMF at 85°C for 2.5h. The reaction isomerised the allyl moiety to the *cis*-prop-1-enyl ether, which was then rendered susceptible to acidic hydrolysis. The $R_f$ value of the 1,4-di-*O*-*cis*-prop-1-enyl derivative **(310)** was identical to that of **(309)**, but treatment of a small quantity of the mixture with acid gave a lower spot on TLC (not recorded). The compound was extracted from the mixture, purified by chromatography and recrystallised from hexane to give compound **(310)** in 83% yield. The $^1$H NMR spectrum showed that the 1,4-di-*O*-*cis*-prop-1-enyl groups had formed because the coupling constants for $CH_3HC{=}CH$-O-Ins were recorded at $\delta = 6.08$ and 6.26ppm and had coupling constants of 6.23 and 6.41Hz respectively, whereas if a *trans*-prop-1-enyl ether had formed the coupling constant would have been in the order of *ca.* 15Hz. The *cis*-prop-1-enyl ether and the *p*-methoxybenzyl group were both acid sensitive so treatment of the derivative with 10% TFA in dichloromethane would cleave both protective groups whilst leaving the benzyl ethers intact. Thus, addition of the compound to the acid resulted in a red-orange solution which was stirred overnight. The only product which was detected by TLC resulted from deprotection of the *p*-methoxybenzyl group which stained purple in the presence of phosphomolybdic acid. The acidic solution was evaporated and the resulting slurry was coevaporated with water and ethanol. A white solid precipitated from the solution and was washed with water, acetone and finally ether to remove any impurities. The solid was crystallised from DMF-ethanol to give 2,5-di-*O*-benzyl-*myo*-inositol **(311)**. Whilst this work was in progress, the same compound was prepared by Gigg and coworkers, [387] but no $^1$H NMR data for the compound were given. Gigg and coworkers prepared 2,5-di-*O*-benzyl-*myo*-inositol by benzylating 1,3,4,6-tetra-*O*-allyl-*myo*-inositol followed by deallylation with palladium on charcoal in the presence of acid. The analytical data (microanalysis) presented by this group [387] was slightly high for the carbon (+0.85%) but the melting point was 270-272°C, and in good agreement with ours (271-273°C).

The $^1$H NMR spectrum of 2,5-di-*O*-benzyl-*myo*-inositol **(311)** is shown in Figure 89 and shows a symmetrical pattern similar to 2,5-di-*O*-methyl-*myo*-inositol. A triplet at $\delta$ = 3.03 for C-5-H (J = 9.16Hz) and a small triplet at $\delta$ = 3.60 for C-2-H (J = 2.57Hz). At $\delta$ = 3.33 there is a ddd, (J = 2.57, 4.77 and 9.71Hz) for C-1-H and C-3-H and at $\delta$ = 3.60 there is a dt (J = 5.13 and 9.34Hz) for C-4-H and C-6-H, both signals integrating for two hydrogens. The two doublets at $\delta$ = 4.75 (J = 4.77Hz) for C-1-OH and C-3-OH and at $\delta$ = 4.83 (J = 5.12Hz) for C-4-OH and C-6-OH both exchanged with $D_2O$. However, the addition of $D_2O$ caused precipitation of the compound which was easily recovered by filtration. A mass spectrum for this compound could not be obtained because of its inherent insolubility in *m*-nitrobenzyl alcohol, glycerol and thioglycerol. 2,5-Di-*O*-

benzyl-*myo*-inositol was used to prepare *myo*-inositol 1,3,4,6-tetrakisphosphorothioate [Ins(1,3,4,6)$PS_4$] **(320)**.

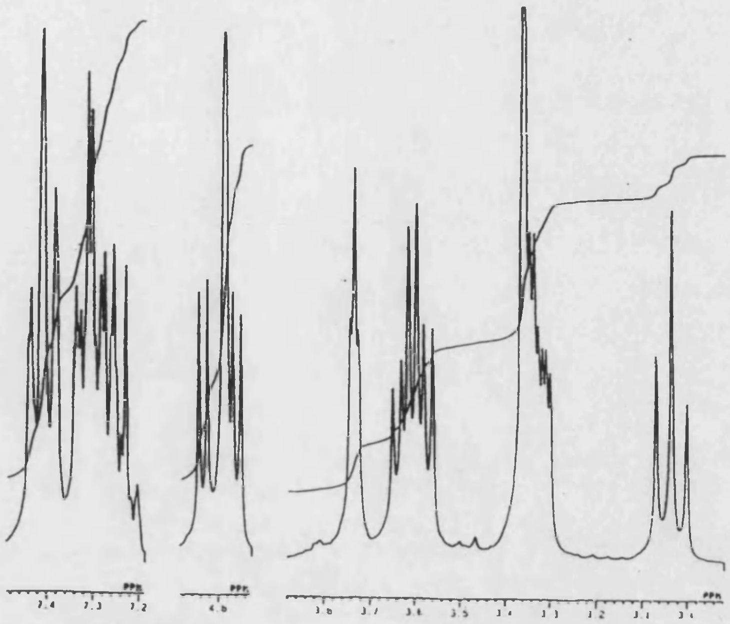


**Figure 89**

### 5.4.2 Synthesis of 2,5-Di-*O*-Methyl *myo*-Inositol 1,3,4,6-Tetrakisphosphate

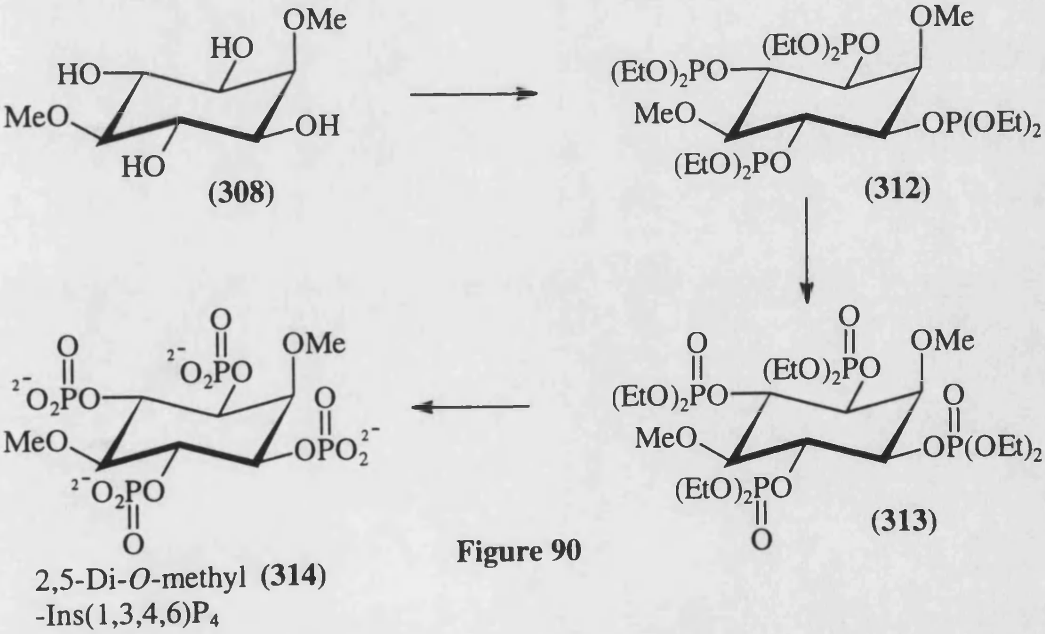



**Figure 90**

2,5-Di-*O*-methyl-*myo*-inositol (**308** in Figure 90) was dissolved in dry DMF and dry *N*,*N*-diisopropylethylamine. The mixture was cooled, diethoxychlorophosphine was added dropwise and and stirred for 45min to give the 1,3,4,6-tetrakisphosphite intermediate **(312)**. Oxidation of the P(III) intermediate was carried out using *t*-butylhydroperoxide to give compound **(313)**. Purification by flash chromatography was not necessary since aqueous work up provided the pure compound as a glass.

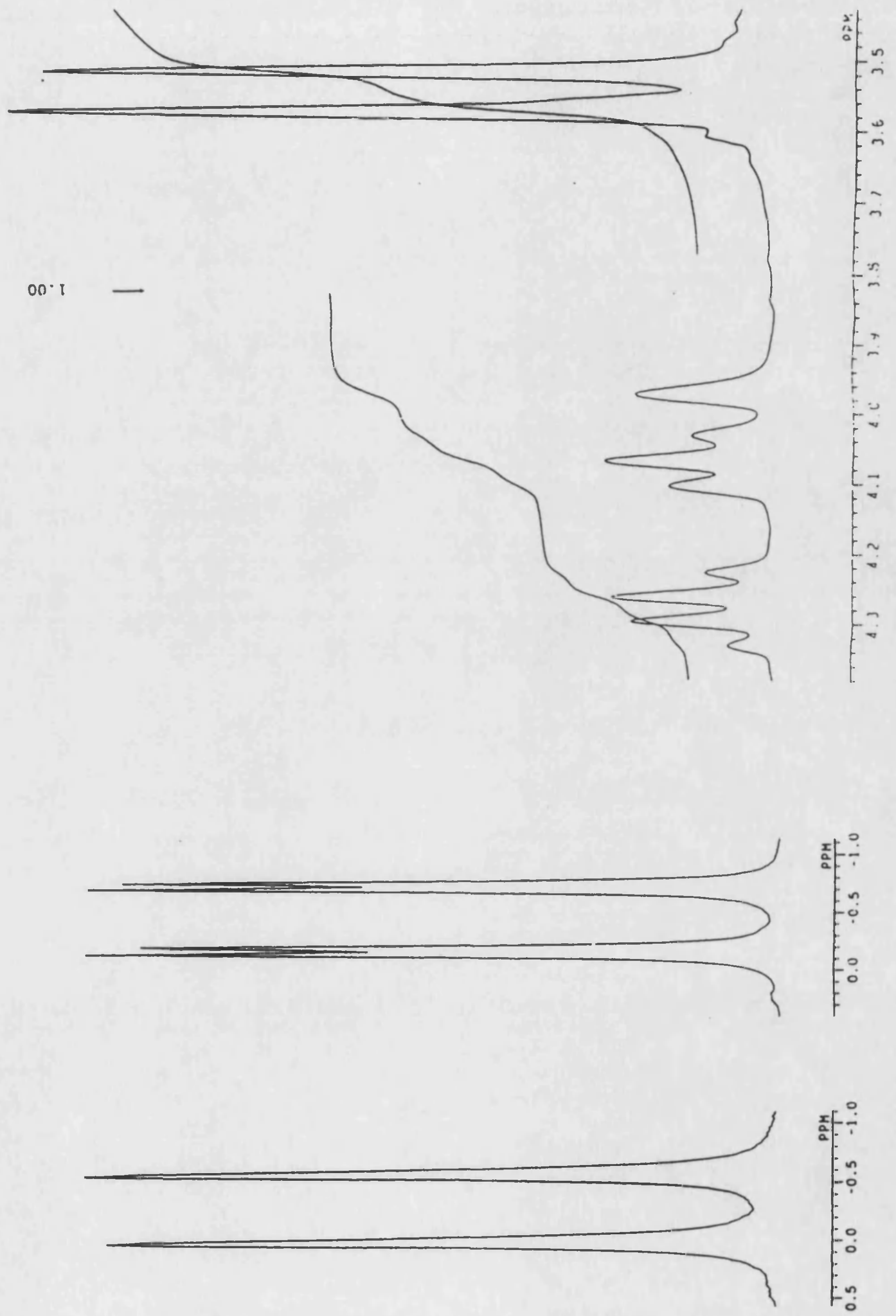


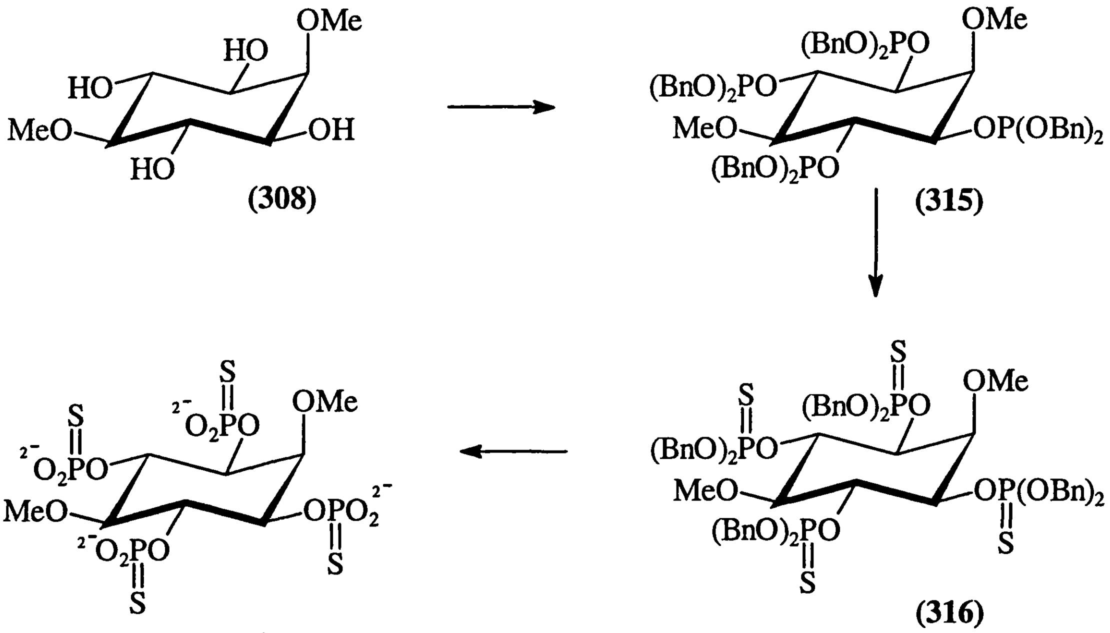
**Figure 91**

The eight ethyl groups were removed from the protected tetrakisphosphate by a two step, one-pot process. Compound **(313)** was dissolved in dry dichloromethane and bromotrimethylsilane was added to the mixture under nitrogen and stirred overnight. In this reaction the eight ethyl groups of compound **(313)** were replaced by trimethylsilyl functions in quantitative yield by $^{31}P$ NMR, in a transetherification process with the production of bromoethane. The solvents were evaporated and water was added to the residue in order to hydrolyse the temporary trimethylsilyl moieties. The final product was purified by ion exchange chromatography on Q-Sepharose Fast Flow using a gradient of 200-1000mM TEAB buffer. The title compound **(314)** eluted at *ca.* 500mM buffer and characterised as its glassy triethylammonium salt.

The $^{1}H$ NMR and $^{31}P$-$^{1}H$ NMR (decoupled and coupled) are given in Figure 91 There were only two peaks in the $^{31}P$-$^{1}H$ (decoupled) spectrum at $\delta = 0.00$ and - 0.58ppm as a consequence of the symmetry through the C-2-H and C-5-H bonds. The $^{31}P$-$^{1}H$ (coupled) spectrum shows two doublets centered at $\delta = 0.00$ (J = 9.9Hz) and - 0.58 (J = 8.0Hz). The $^{1}H$ NMR spectrum shows two methyl groups at $\delta = 3.51$ and 3.57 together with the C-5-H which was hidden under the methyl singlet at $\delta = 3.57$. C-2-H was a broad singlet at $\delta = 3.97$ and C-1-H and C-3-H were as a triplet at $\delta = 4.06$ (J = 9.52Hz) as well as C-4-H and C-6-H being identified as a quartet at $\delta = 4.28$ (J = 9.40Hz). A sample of the compound gave a satisfactory accurate -ve FAB mass spectrum.

### 5.4.3 Synthesis of 2,5-Di-*O*-Methyl *myo*-Inositol 1,3,4,6-Tetrakisphosphorothioate

The reagent, bis(benzyloxy)diisopropylaminophosphine **(96)** was used to phosphitylate 2,5-di-*O*-methyl-*myo*-inositol (**308** in Figure 92). A mixture of 1*H*-tetrazole and bis(benzyloxy)diisopropylaminophosphine was stirred in DMF for 1h to give the tetrazolide. The formation of this complex in DMF was somewhat slower than in dichloromethane, with the appearance of two peaks at $\delta = +126.12$ and $\delta = +127.47$ppm in the $^{31}P$-$^{1}H$ NMR (decoupled) spectrum. The tetrol was then added to the tetrazolide complex and the mixture was stirred for a further 2h. The $^{31}P$-$^{1}H$ NMR (decoupled) spectrum at this stage showed two peaks centered at $\delta = +141.26$ and $\delta = +139.45$ppm which indicated a phosphitylated *myo*-inositol derivative. However, at high resolution, a single AB spin coupling pattern was observed (**315** in Figure 93) with a $^{5}J_{PP} = 3.90$Hz. Sulphoxidation of this intermediate with sulphur $S_8$, in (pyridine-DMF, 1:2), gave the protected intermediate **(316)** within 15min. This was the first time that sulphoxidation was carried out in this mixture which was an improvement on sulphur in pyridine only. The technique was improved for the synthesis of other phosphorothioates, *e.g.* Ins(1,2,4,5)$PS_4$, which was discussed previously, together with all phosphorothioates which follow. The sulphur was filtered off and the solvent carefully evaporated *in vacuo*. The protected phosphorothioate **(316)** was purified by flash chromatography and isolated in 82% yield as a clean syrup. The $^{31}P$-$^{1}H$ NMR (coupled and decoupled) spectra for **(316)** are shown in Figure 94. This shows two singlets (decoupled) at $\delta$ = 67.09 and $\delta$ = 69.11ppm, which was the area observed for protected phosphorothioates. The $^{31}P$-$^{1}H$ NMR coupled spectrum shows two doublet of quintets, (J = 8 and 9.9Hz, and J = 8 and 9.9Hz).




2,5-Di-*O*-methyl **(317)**
-Ins(1,3,4,6)$PS_4$

**Figure 92**

The octabenzyl derivative **(316)** was deprotected with sodium in liquid ammonia followed by purification on Q-Sepharose eluting at *ca.* 800mM TEAB buffer in 25% yield and was characterised as the triethylammonium salt. The product **(317)** exhibited a similar $^{1}H$ NMR spectrum to the phosphate derivative **(314)** and the $^{31}P$-$^{1}H$ NMR spectra (in Figure 95) shows two singlets at $\delta$ = 46.7 and $\delta$ = 48.8ppm, for the $^{31}P$-$^{1}H$-decoupled spectrum, and the inset $^{31}P$-$^{1}H$-coupled spectrum shows two doublets (J = 10.1 and 10.1Hz) respectively.

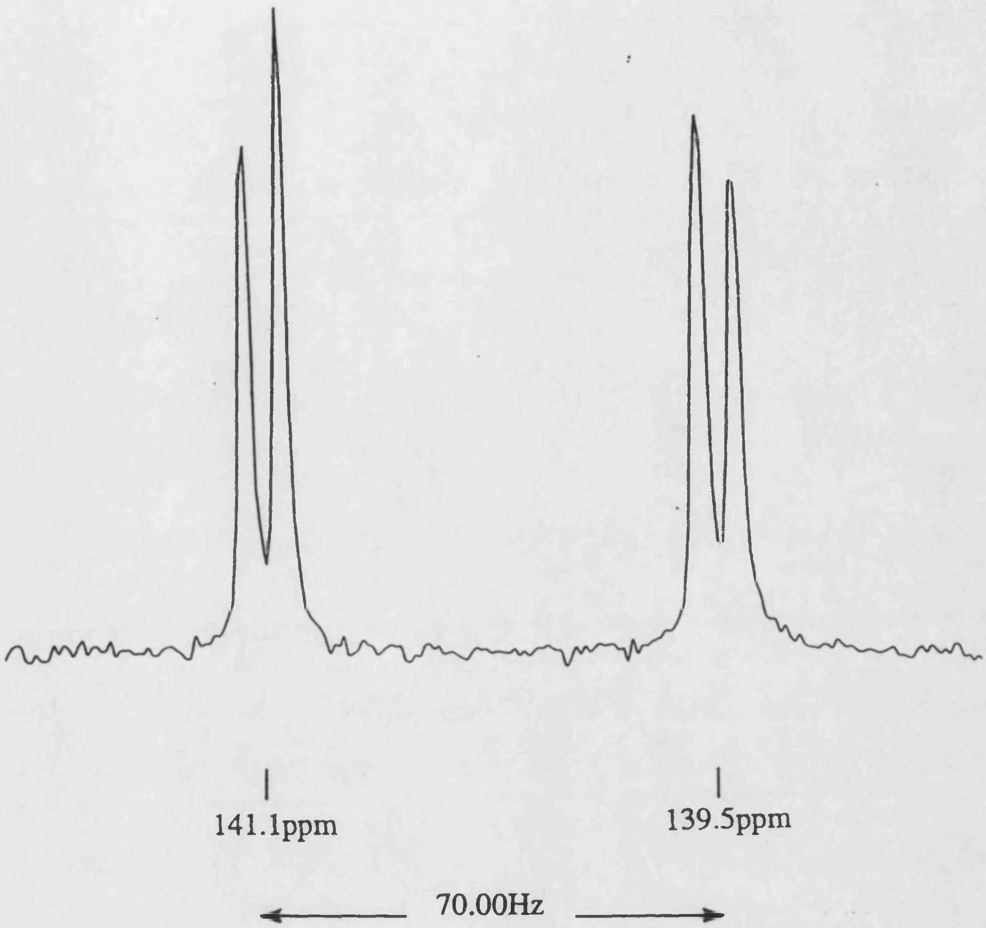



**Figure 93**

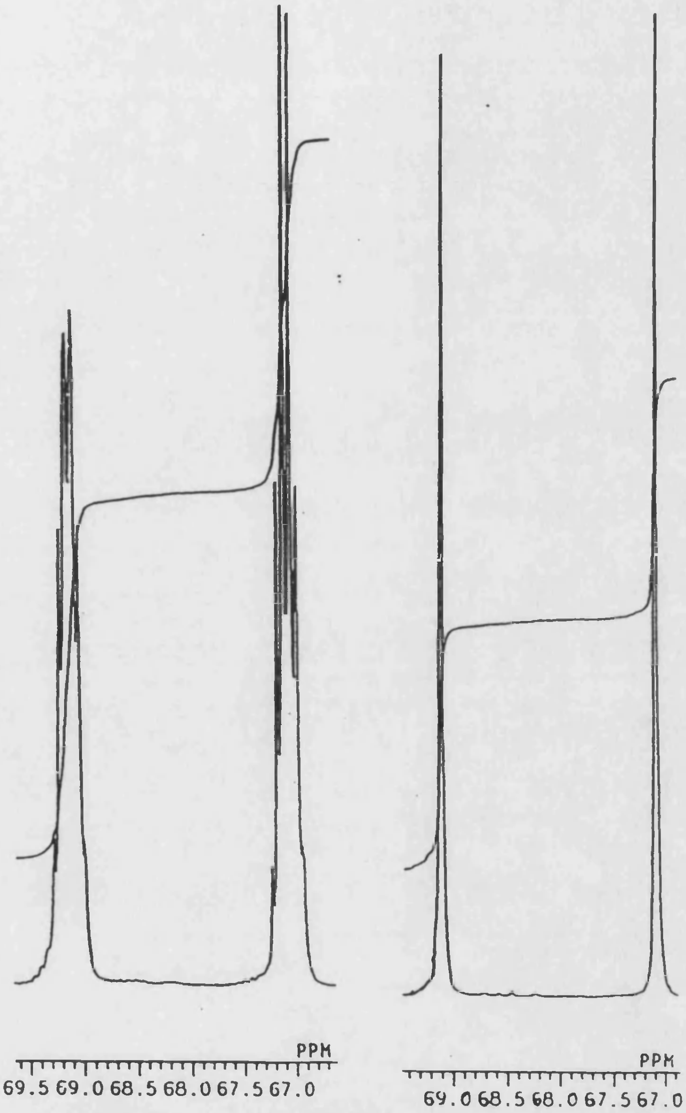



**Figure 94**

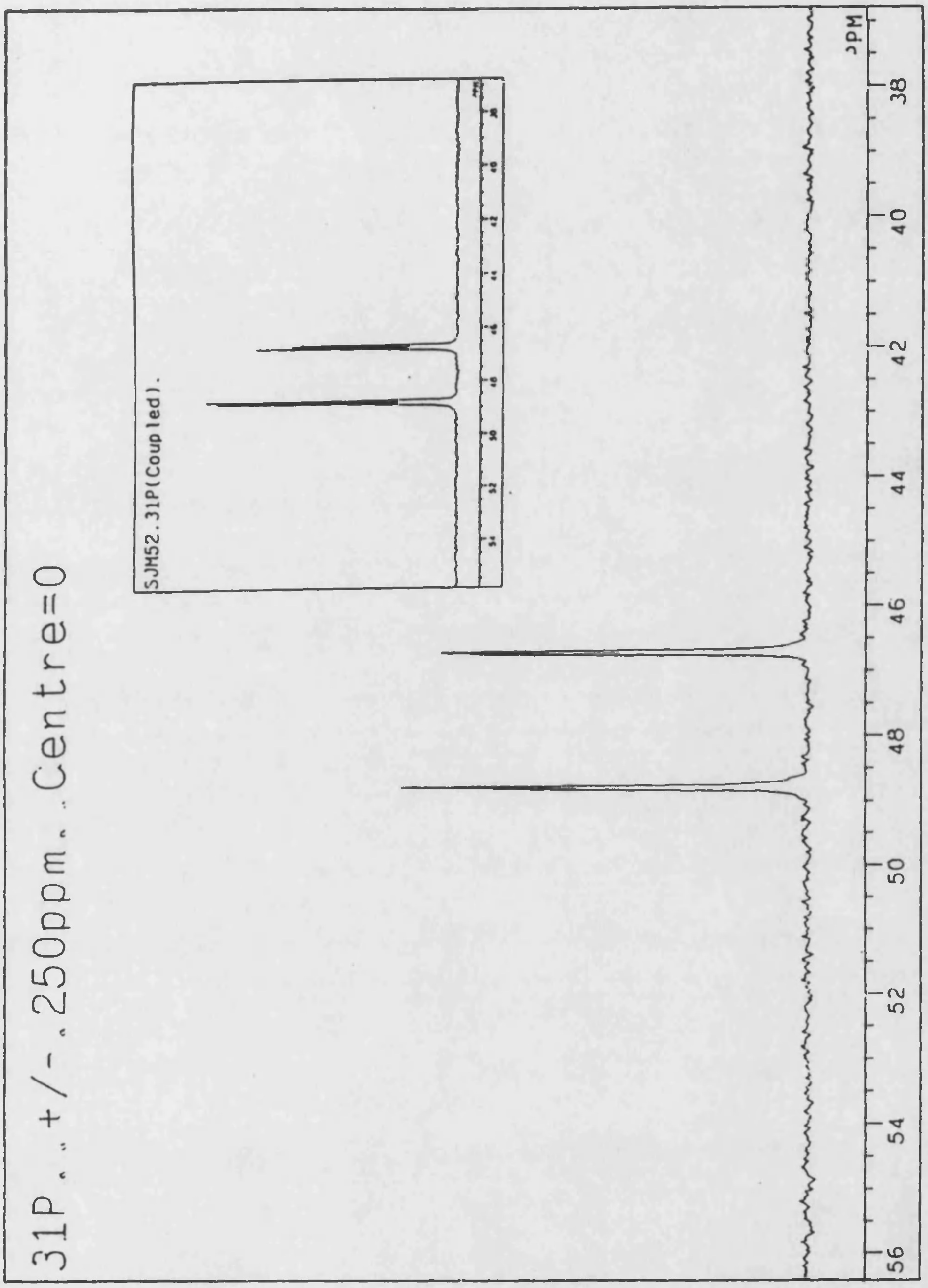

31P..+/-..250ppm..Centre=0
SJM52.31P(Coupled).
PPM
56
54
52
50
48
46
44
42
40
38

Figure 95

### 5.4.4 Synthesis of *myo*-Inositol 1,3,4,6-Tetrakisphosphorothioate

A mixture of the phosphitylating reagent **(96)** and 1*H*-tetrazole was stirred in dry DMF for 1h. 2,5-Di-*O*-benzyl-*myo*-inositol (**311** in Figure 96) was added to the mixture and stirred for 1h. A similar AB spin coupling pattern to compound **(315)** was observed with $^{5}J_{PP}$ = 4.27Hz. Sulphur and pyridine-DMF (1:2) were added, and the mixture was stirred for 10min. The sulphur was filtered and the solvents were evaporated *in vacuo.* The residue was purified by chromatography to give the decabenzyl derivative **(319)** in 71% yield.

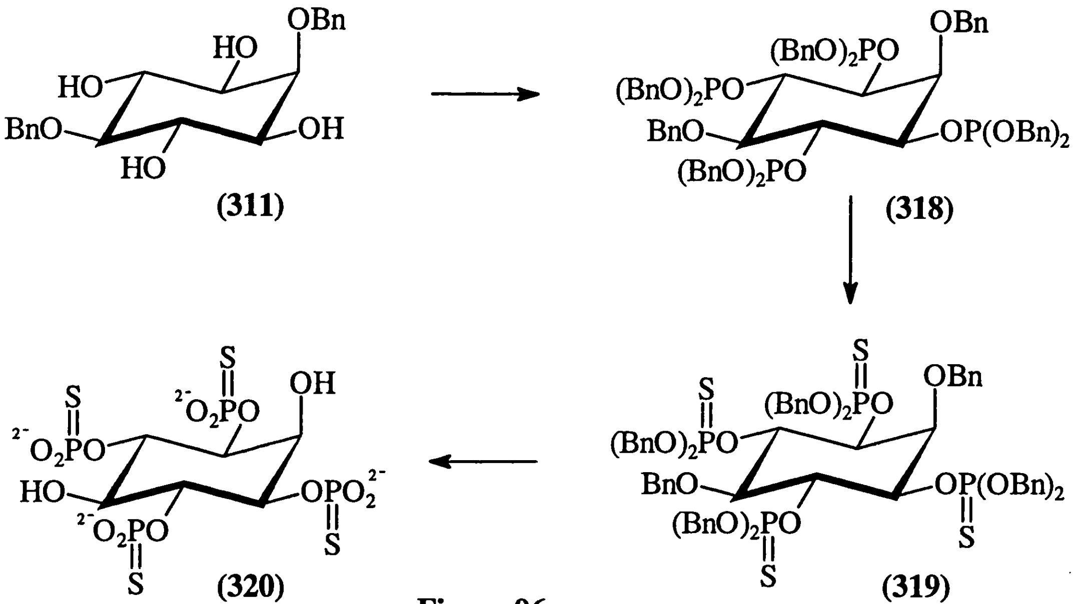



**Figure 96**

Deprotection of the compound with sodium in liquid ammonia followed by purification by ion exchange chromatography gave Ins(1,3,4,6)PS$_4$ **(320)** in 46% yield. The $^{31}$P-$^{1}$H NMR spectra were similar to the 2,5-di-*O*-methyl derivative **(317)** except the chemical shifts were slightly different; $\delta$ = 46.7 (d, J = 12.2Hz) and $\delta$ = 42.4ppm, (d, J = 9.8Hz).

### 5.4.5 Pharmacology

The three *meso* compounds, 2,5-di-*O*-methyl Ins(1,3,4,6)P$_4$ **(314)** 2,5-di-*O*-methyl Ins(1,3,4,6)PS$_4$ **(317)** and Ins(1,3,4,6)PS$_4$ **(320)** have been evaluated. [422] It is known that Ins(1,3,4,6)P$_4$ is a partial agonist with high intrinsic activity [216] mobilising 85% of the $^{45}Ca^{2+}$ mobilised by Ins(1,4,5)P$_3$ from electrically permeabilised SH-SY5Y human neuroblastoma cells with an $EC_{50}$ value of 19.6μM. Methylating the hydroxyl groups of Ins(1,3,4,6)P$_4$ gave 2,5-di-*O*-methyl Ins(1,3,4,6)P$_4$ **(314)** and removed all $^{45}Ca^{2+}$-

mobilising activity in a fashion similarly observed for 3,6-di-*O*-benzoyl Ins(1,2,4,5)$P_4$ **(276)**. This result indicated the importance of one or both hydroxyl groups which were critical for interacting with the Ins(1,4,5)$P_3$ receptor. Similarly, substitution of all the phosphates with phosphorothioate functions gave 2,5-di-*O*-methyl Ins(1,3,4,6)$PS_4$ **(317)** and Ins(1,3,4,6)$PS_4$ **(320)** [from Ins(1,3,4,6)$P_4$] which were poor agonists at the Ins(1,4,5)$P_3$ receptor, mobilising <15% of the maximal dose of Ins(1,4,5)$P_3$ at 30μM. The important information here is that none of these compounds antagonised the $^{45}Ca^{2+}$-release activity of Ins(1,4,5)$P_3$.

The three compounds were also tested for their interaction with the enzymes 3-kinase from CRBHS and 5-phosphatase from HEG. For comparison Ins(1,4,5)$P_3$ had a $K_m$ values of 0.85μM and 13.8μM for 3-kinase and 5-phosphatase respectively. Both 2,5-di-*O*-methyl derivatives **(314)** and **(317)** were poorly recognised by the 3-kinase with $K_i$ values of >100μM and 105μM respectively. However, Ins(1,3,4,6)$PS_4$ was recognised by 3-kinase with a $K_i$ value of 46μM. This value was similar to *scyllo*-Ins(1,3,4,6)$PS_4$ [414] which had a $K_i$ value of 56.7μM and was discussed in Chapter 4. Ins(1,3,4,6)$PS_4$ had an increased affinity for 5-phosphatase with a $K_i$ value of 1.9μM compared to Ins(1,3,4,6)$P_4$ with $K_i$ value of 7.7μM. Methylation at the 2- and 5-positions gave compounds **(314)** and **(317)** which had $K_i$ values of 15.9μM and 1.4μM respectively. Thus tetrakisphosphates, generally have a higher affinity for 5-phosphatase over trisphosphates and replacing phosphates with phosphorothioates resulted in increased affinity for 5-phosphatase from HEG.

### 5.5 Introduction to the Synthesis of DL-Ins(1,4,6)$P_3$ and DL-Ins(1,4,6)$PS_3$

D-Ins(1,4,6)$P_3$ may be derived from Ins(1,3,4,6)$P_4$ by removing the phosphate group at the 3-position to give a compound with a pseudo D-1,4,5 motif with inverted hydroxyl groups at the pseudo 2- and 3-positions, as described in Chapter 4. First, DL-Ins(1,4,6)$P_3$ was synthesised in order to establish any properties of $Ca^{2+}$-release, followed by its phosphorothioate, DL-Ins(1,4,6)$PS_3$ **(336)** which proved to be a promising lead in the development of a full antagonist. After the synthesis of the racemic mixture, the individual enantiomers were successfully made using (*S*)-(+)-*O*-acetylmandelic acid which was used to resolve a precursor to D-**(346)** and L-2,3,5-tri-*O*-benzyl-*myo*-inositol **(345)**.

### 5.5.1 The Synthesis of DL-1,2,5-Tri-*O*-Benzyl-*myo*-Inositol

DL-3,6-Di-*O*-allyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(303)** was used as the starting material for the synthesis of DL-1,2,5-tri-*O*-benzyl-*myo*-inositol **(328)**. For this racemic synthesis, all the intermediates are drawn in the L-configuration so the phosphorylation precursor **(328)** is of D-configuration (in Figure 97).

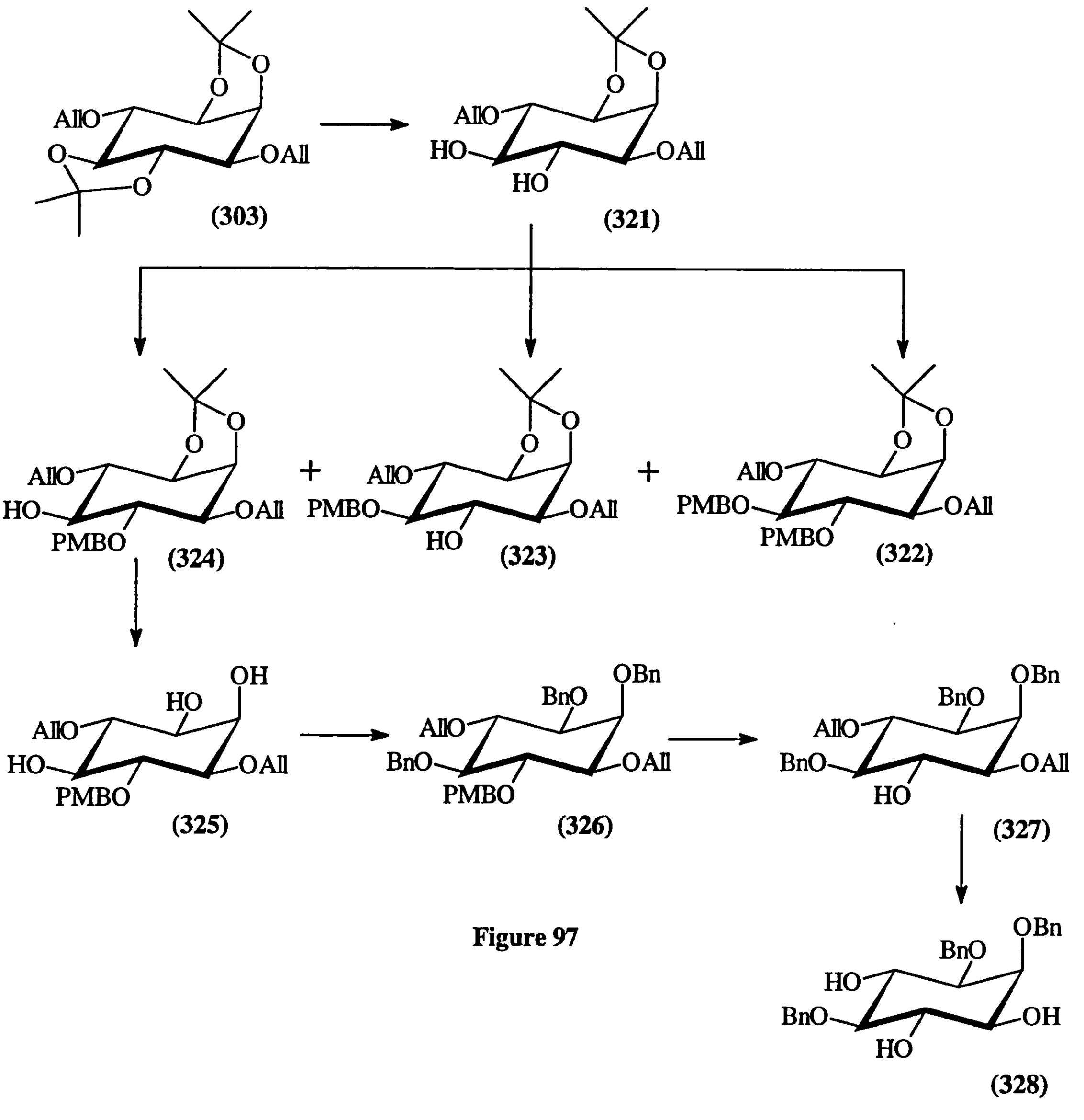



**Figure 97**

DL-3,6-Di-*O*-allyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(303)** was prepared from DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(37)** by allylating the 3- and 6-hydroxyl groups with allyl bromide and sodium hydride in DMF. The less stable *trans* isopropylidene acetal was removed selectively using a catalytic amount of toluene-*p*-sulphonic acid and one equivalent of ethane 1,2-diol, in dichloromethane with stirring. The temperature was kept at 0°C (with ice) for 10min after which the cooling was removed and the reaction mixture was allowed to warm to room temperature whilst stirring. It was found that the nature of the protective group at positions 3- and 6-, influenced the rate of hydrolysis of the 4,5-*trans* isopropylidene acetal. If ester groups were present, the rate of hydrolysis of the *trans* acetal was slow. When positions 3- and 6- were blocked with benzyl groups the rate of hydrolysis was smooth and complete within 45min, whereas cooling was necessary when the allyl group occupied these positions, in order to prevent the formation of excess DL-1,4-di-*O*-allyl-*myo*-inositol **(304)** and therefore to control the formation of DL-1,4-di-*O*-allyl-2,3-*O*-isopropylidene-*myo*-inositol. When the solution became cloudy (after 40min), triethylamine was added to quench the reaction and water was added to the solution. DL-1,4-Di-*O*-allyl-*myo*-inositol **(304)** was soluble in water and a small amount of starting material **(303)** and product **(321)** were present in the organic layer. The organic solvent was evaporated to give a solid and the mixture was recrystallised from ethyl acetate-hexane to give pure DL-1,4-di-*O*-allyl-2,3-*O*-isopropylidene-*myo*-inositol **(321)** in 68% yield. No starting material was detected after recrystallisation because it was soluble in all organic solvents at room temperature.

The *trans* diol was now exposed and it was envisaged that the 6-position would be more reactive towards tin-mediated alkylation over the 5-position. Thus, treatment of the diol **(321)** with a mixture of dibutyltin oxide, tetrabutylammonium iodide, *p*-methoxybenzyl chloride and acetonitrile in the presence of 4Å sieves (in a Soxhlet) for 48h at reflux temperature gave three products. The major product was found to be DL-1,4-di-*O*-allyl-2,3-*O*-isopropylidene-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(324)** $R_f = 0.40$ (ether-hexane, 3:2) which was isolated in 52% yield. The $^1H$ NMR spectra in $CDCl_3$ distinctly shows the hydroxyl at the 5-position coupling to the ring hydrogen, $\delta = 3.43$, as a ddd, (J = 2.02, 8.24 and 8.24Hz) and upon $D_2O$ exchange the ddd collapsed to a dd, (J = 8.24 and 8.24Hz). C-5-H is usually the most upfield hydrogen and thus the C-6-OH has been benzylated. Also formed was DL-1,4-di-*O*-allyl-2,3-*O*-isopropylidene-5-*O*-*p*-methoxybenzyl-*myo*-inositol **(323)** $R_f = 0.22$ (ether-hexane, 3:2), isolated in 31% yield. The $^1H$ NMR spectra showed coupling of C-6-H to its hydroxyl, at $\delta = 3.94$, as a dt, (J = 1.46 and 9.71Hz) and upon $D_2O$ exchange the dt collapsed to a triplet (J = 9.53Hz), thus C-5-OH was alkylated. At $R_f = 0.50$ (ether-hexane, 3:2), DL-1,4-di-*O*-allyl-2,3-*O*-isopropylidene-5,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol **(322)** was isolated in 14% yield. Surprisingly, the only crystalline solid was the 5-*O*-*p*-methoxybenzyl derivative, which formed crystals several centimetres in length which could be recrystallised from hexane, (m.p. 70-72°C). The three products were easily separated by flash chromatography using the same solvent system as for the TLC system.

The major compound was treated in one of two ways in order to obtain the required DL-1,2,5-tri-*O*-benzyl-*myo*-inositol **(328)**. First, the 2,3-*O*-isopropylidene was hydrolysed under acidic conditions, using methanol-1M HCl (9:1) which was kept at 50°C for 30min. Under these mild acidic conditions the *p*-methoxybenzyl group was not affected and DL-1,4-di-*O*-allyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(325)** was isolated in 80% yield after crystallisation from ethyl acetate-hexane. The three hydroxyl groups at positions 2-, 3- and 5-, were alkylated with benzyl bromide using sodium hydride as base in DMF. Work up and purification by column chromatography gave a syrup which was dissolved in hot hexane and crystallised on cooling to give the fully protected compound, DL-1,4-di-*O*-allyl-2,3,5-tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(326)** in 85% yield (m.p. 53-54°C).

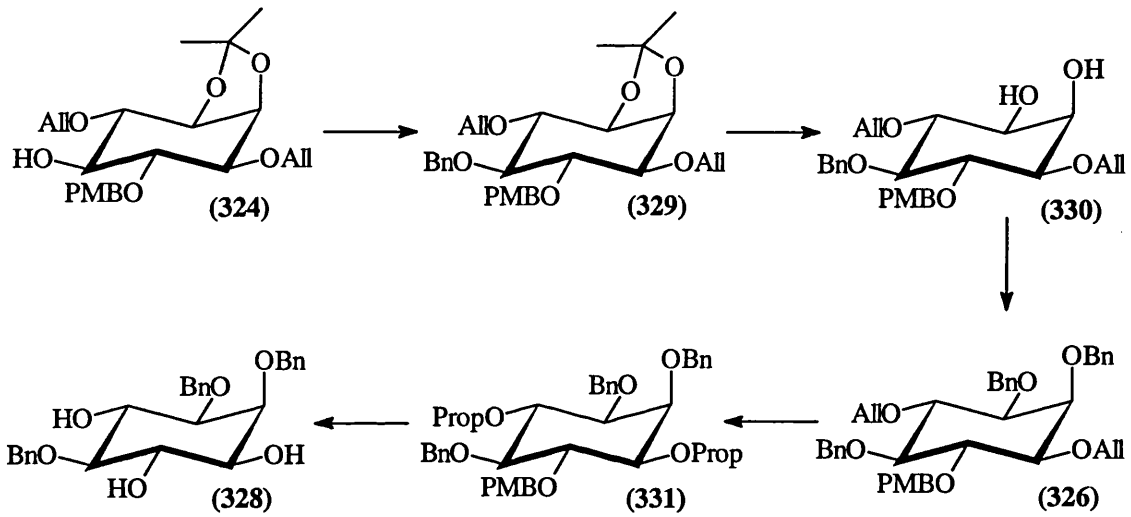



**Figure 98**

DL-1,4-Di-*O*-allyl-2,3-*O*-isopropylidene-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(324** in Figure 98) was benzylated with benzyl bromide and sodium hydride in DMF to afford the totally protected compound DL-1,4-di-*O*-allyl-5-*O*-benzyl-2,3-*O*-isopropylidene-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(329)** as a syrup, in 93% yield, after column chromatography. The *cis* 2,3-acetal was removed with methanol-1M HCl (9:1), at 50°C for 30min. Work up and crystallisation (from ether-hexane) gave DL-1,4-di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(330)** (m.p. 87-88°C) in 88% yield. This compound had a *cis* 1,2-diol and was ideal for resolution at this stage using (*S*)-(+)-*O*-acetylmandelic acid and will be described later. The hydroxyl groups were benzylated at the 2-, 3- and 5-positions to give the known, fully protected DL-1,4-di-*O*-allyl-2,3,5-tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(326)**. This compound was then deprotected in one of two ways, the latter being the most successful. First, a two step deprotection strategy was employed using DDQ (in dichloromethane-water, 15:1) in order to cleave the *p*-methoxybenzyl group. An interesting colour change took place. Initially the colour of DDQ was mustard-yellow which immediately turned green on contact with the solvent mixture. As the reaction proceeded, the solution changed to a red-brown colour. After *ca.* 1h the solution was filtered and the precipitate was salmon-pink in colour. The distinctive odour of *p*-methoxybenzaldehyde was another positive sign that the *p*-methoxybenzyl ether had been cleaved by DDQ. All other protective groups were intact to give, after work up, DL-1,4-di-*O*-allyl-2,3,5-tri-*O*-benzyl-*myo*-inositol **(327)** in 87% yield as a syrup. The two allyl groups were removed in a one pot deprotection reaction using 10% palladium on charcoal, together with toluene-*p*-sulphonic acid in an ethanol-water mixture at the reflux temperature for 5h. Unfortunately the mixture was refluxed for too long, and other debenzylated products present on the baseline were observed by TLC. Deallylation with palladium on carbon gave DL-1,2,5-tri-*O*-benzyl-*myo*-inositol **(328)** in 40% yield after work up and chromatography.

Second, the allyl groups at the 1- and 4-positions were isomerised with potassium *t*-butoxide in dry DMSO to give the *cis* 1,4-di-*O*-prop-1-enyl derivative **(331)** in 80% yield after work up and purification by chromatography. For this reaction the isomerisation was easy to follow because the starting material ($R_f$ = 0.50, in ether-hexane, 1:1) had a lower $R_f$ value than the product ($R_f$ = 0.72, in ether-hexane, 1:1). The melting point (89-91°C) was also higher than for the allyl derivative, and generally, if one allyl moiety is isomerised to a *cis*-prop-1-enyl group the melting point increases by 15-20°C. The smell of DMSO was still apparent even after chromatography and difficult to remove, however, satisfactory analytical data was achieved for the product DL-2,3,5-tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-1,4-di-*O*-*cis*-prop-1-enyl-*myo*-inositol **(331)**. The next step was was a one pot acidic hydrolysis of the *cis*-prop-1-enyl and the *p*-methoxybenzyl ethers. Thus treatment of compound **(331)** with ethanol-1M aqueous HCl (2:1) for 4h hydrolysed the acid sensitive protecting groups. The solvents were evaporated *in vacuo* and were purified by column chromatography to give DL-1,2,5-tri-*O*-benzyl-*myo*-inositol **(328)**.

The $^1$H NMR spectrum of DL-1,2,5-tri-*O*-benzyl-*myo*-inositol in $CDCl_3$ is shown in Figure 99. The three hydroxyl signals are very broad, $\delta$ = 1.65-2.65, but the hydrogens of the *myo*-inositol ring are well separated as shown on the expansion. The C-5-H is a triplet and is deshielded most $\delta$ = 3.23, (J = 9.16Hz). The two dd, at $\delta$ = 3.30 (J = 2.39 and 9.71Hz) and $\delta$ = 3.40 (J = 2.57 and 9.71Hz) are C-1-H and C-3-H. The two triplets at $\delta$ = 3.82 (J = 9.52Hz) and $\delta$ = 4.12 (J = 9.52Hz) are C-4-H and C-6-H, whilst the unique triplet at δ = 4.06 is C-2-H. The 2-D COSY spectrum was most informative when it was run in $d_6$-DMSO, (Figure 100). The hydroxyl groups were coupled to the ring hydrogens of DL-1,2,5-tri-*O*-benzyl-*myo*-inositol **(328)**. Since the compound was racemic, the tri-*O*-benzyl compound will be named DL-1,2,5-tri-*O*-benzyl-*myo*-inositol, therefore the hydroxyl groups at positions 3-, 4- and 6 are exposed. The triplet at δ = 3.97 is C-2-H because of the small coupling between two axial-equatorial positions. The signal furthest upfield is a triplet at δ = 3.09 (J = 9.15Hz) and the C-1-H is a dd, at δ = 3.29, because the hydroxyl group is not exposed and there is no extra coupling in $d_6$-DMSO. The ddd, at δ = 3.33 corresponds to position C-3-H because of the extra coupling in $d_6$-DMSO and has cross peaks with the dt, corresponding to C-4-H at δ = 3.64 and finally C-6-H is also a dt, at δ = 3.80. Both these dt signals gave cross peaks with the triplet corresponding to C-5-H. Thus the correct hydroxyl positions are exposed for phosphitylation to give DL-Ins(1,4,6)$P_3$ **(334)** and DL-Ins(1,4,6)$PS_3$ **(336)**.

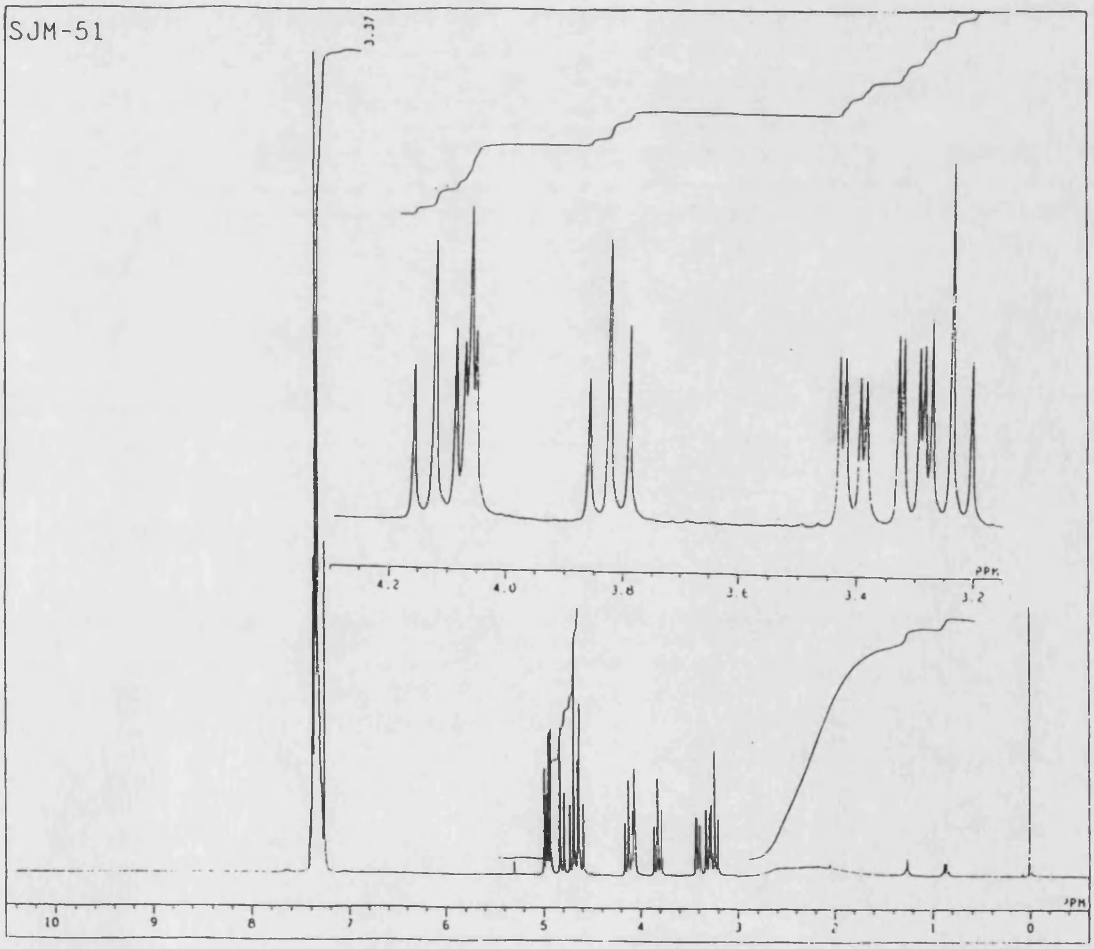



**Figure 99**

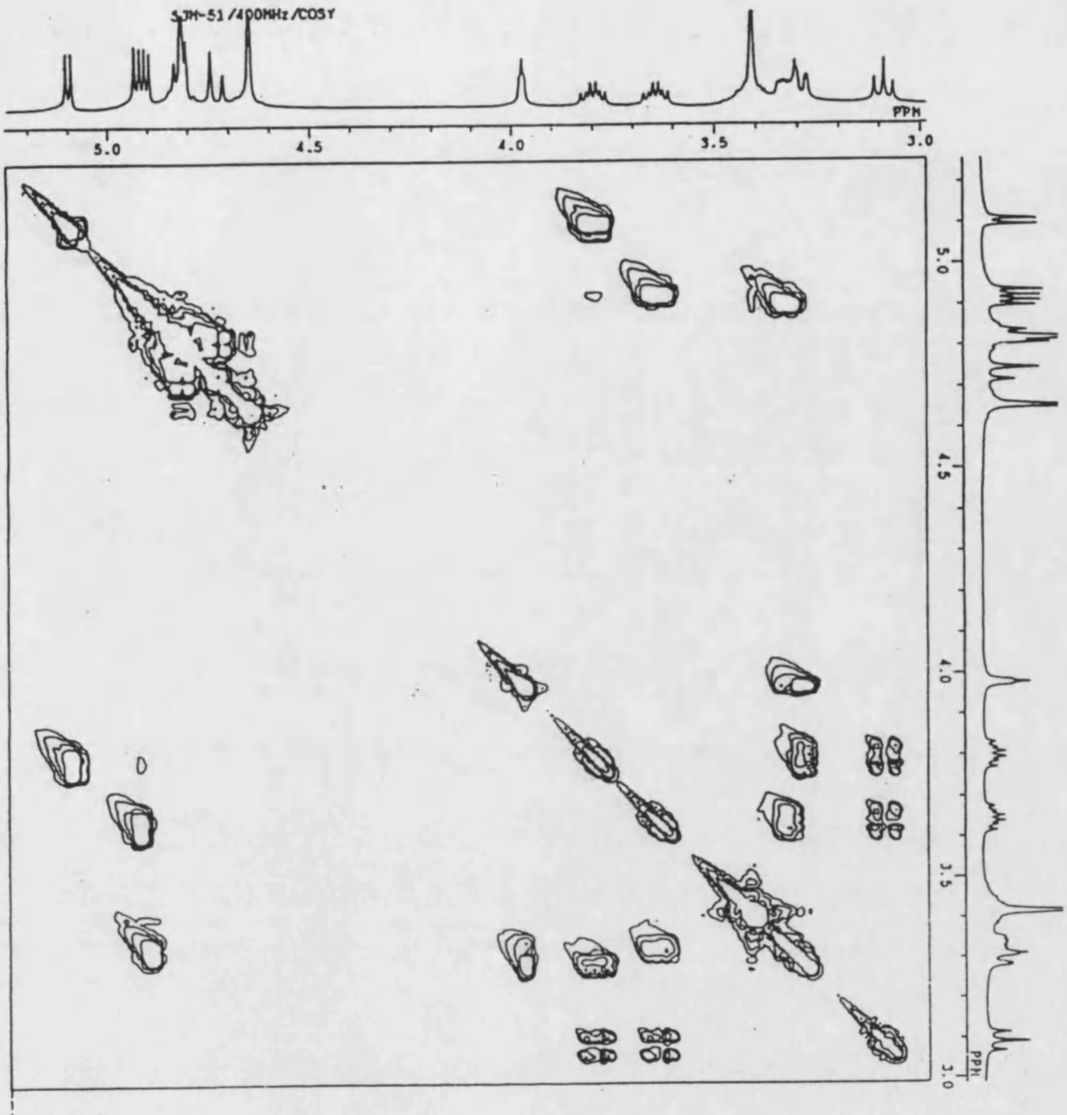



**Figure 100**

### 5.5.2 Synthesis of DL-Ins(1,4,6)$P_3$

The treatment of bis(benzyloxy)diisopropylaminophosphine **(96)** with 1*H*-tetrazole in dry dichloromethane gave the tetrazolide complex **(290)** indicated by the $^{31}$P NMR spectrum at $\delta$ = +126.73ppm. The addition of DL-1,2,5-tri-*O*-benzyl-*myo*-inositol **(328)** to the tetrazolide produced the familiar AB spin coupling pattern together with a singlet at $\delta$ = +141.6ppm for the phosphitylated C-4-OH. The AB system was assigned to the phophitylated 1,6-bisphosphite triester system ($\delta$ = +140.2 and +142.3ppm, $^5J_{PP}$ = 3.66Hz) in Figure 101. The AB system implies phosphitylation of the vicinal diol and the presence of unreacted tetrazolide complex indicated complete phosphitylation, since the tetrazolide complex is very reactive toward nucleophiles, including water. Oxidation of the trisphosphite triester with *t*-butylhydroperoxide gave the fully protected derivative, DL-1,2,5-tri-*O*-benzyl-3,4,6-tris[di(benzyloxyphospho)]-*myo*-inositol **(333)** as a syrup in 85% yield after work up and purification by flash chromatography. The important step in the work up was the wash with sodium metabisulphite solution which removed the excess *t*-butylhydroperoxide. The compound was deprotected using sodium in liquid ammonia. The crude product was purified by ion exchange chromatography on Q-Sepharose fast flow using a gradient of TEAB as eluent to give pure DL-Ins(1,4,6)$P_3$ (**334** in Figure 102) in 60% yield as its glassy triethylammonium salt. The $^{31}P$ and $^{1}H$ NMR data will be discussed for the chiral compound. The accurate -ve FAB was also satisfactory.

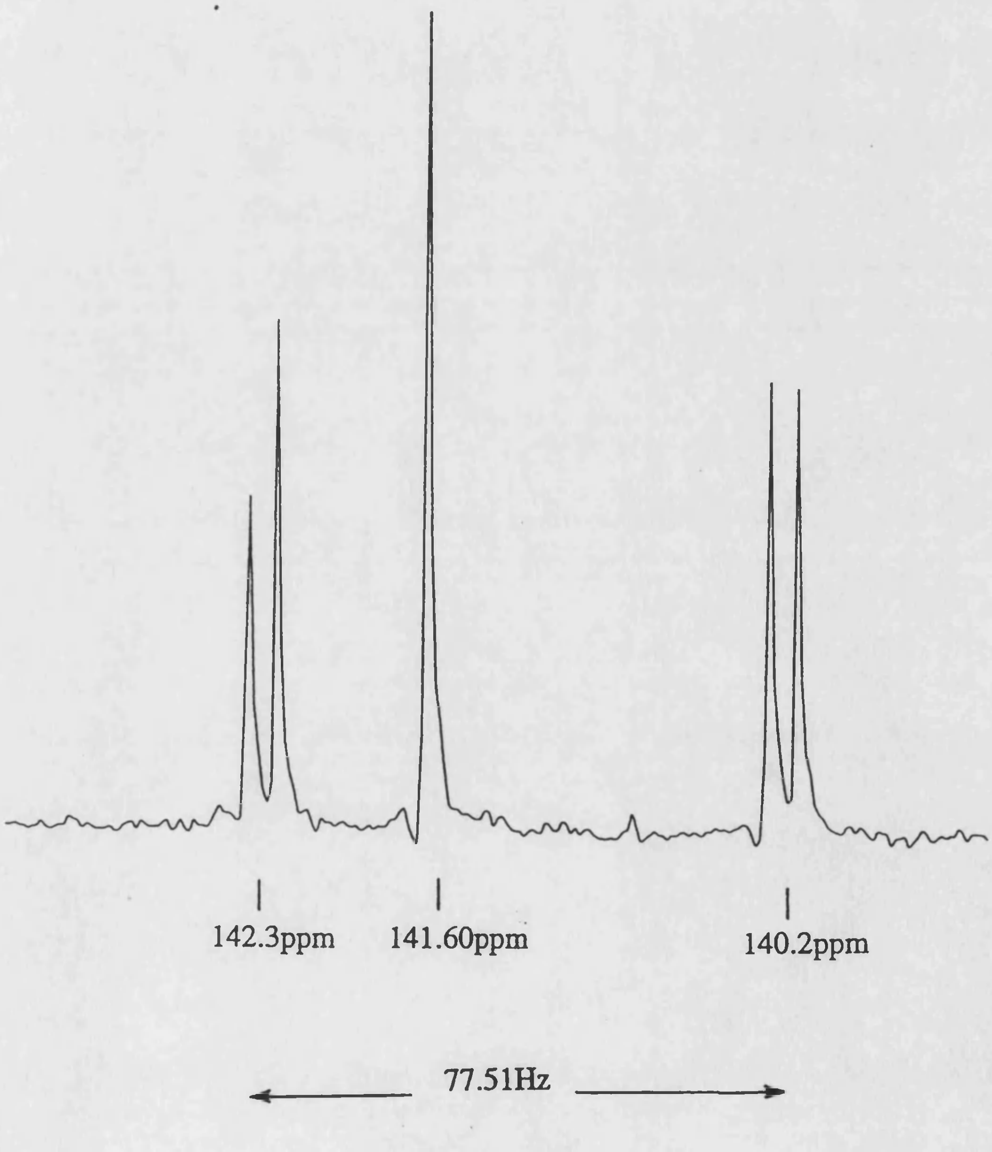
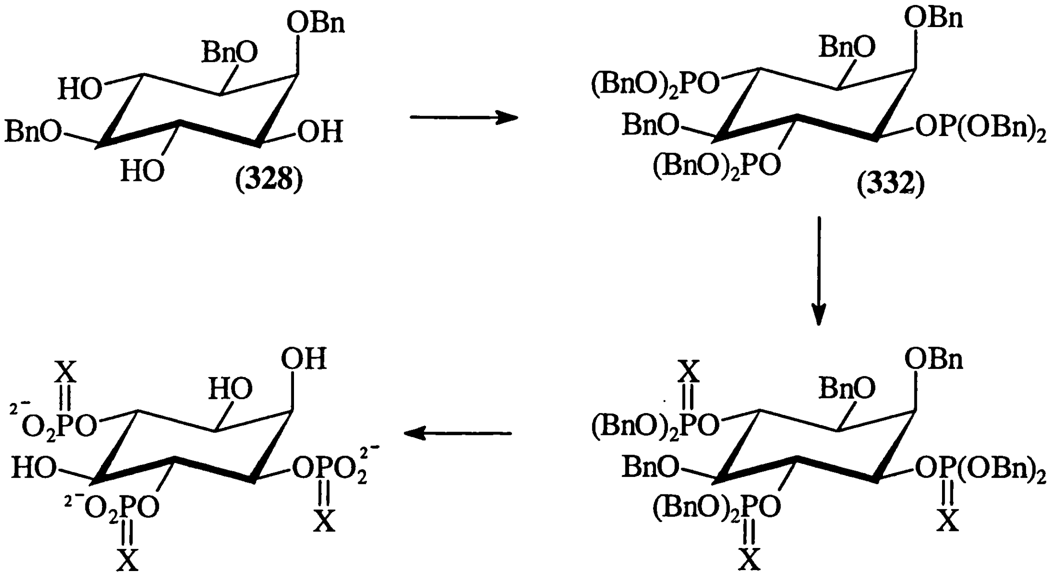



**Figure 101**




DL-Ins(1,4,6)$P_3$ X = O **(334)**

DL-Ins(1,4,6)$PS_3$ X = S **(336)**

X = O **(333)**

X = S **(335)**

**Figure 102**

### 5.5.3 Synthesis of DL-Ins(1,4,6)$PS_3$

The tetrazolide complex was formed in the same way as for the synthesis of DL-Ins(1,4,6)$P_3$ and the triol was added to the tetrazolide **(290)** in the same way in order to form the trisphosphite triester **(332)** with the formation of the familiar AB spin coupling pattern. However, at this stage the dichloromethane was evaporated and the syrup was dissolved in dry DMF (2ml) and dry pyridine (1ml), followed by the addition of sulphur (2 equivalents per phosphite). The reaction was complete within 5min, the excess sulphur was filtered, and the solvents were evaporated *in vacuo.* Work up and purification by flash chromatography provided the fully protected DL-1,2,5-tri-*O*-benzyl-3,4,6-tris[di(benzyloxyphosphorothio)]-*myo*-inositol **(335)** as a sulphur free syrup in 81% yield. Deprotection of the fully blocked compound with sodium in liquid ammonia followed by purification by ion exchange chromatography over Q-Sepharose Fast Flow, eluting with a gradient of TEAB buffer at *ca.* 600mM to give DL-Ins(1,4,6)$PS_3$ (**336** in Figure 102) as the triethylammonium salt. The $^{31}$P and $^{1}$H NMR data will be discussed for the chiral compound.

### 5.5.4 Pharmacology

DL-Ins(1,4,6)$P_3$ and DL-Ins(1,4,6)$PS_3$ have been evaluated for their $Ca^{2+}$-releasing properties. However, Ozaki and coworkers have tested D-Ins(1,4,6)$P_3$ for $Ca^{2+}$-release and its interaction with 3-kinase and 5-phosphatase, which was described in Chapter 4.

DL-Ins(1,4,6)$P_3$ was evaluated as a $Ca^{2+}$-mobilising agonist in permeabilised rabbit platelets, in comparison to Ins(1,4,5)$P_3$ and Ins(1,3,4,6)$P_4$. Ozaki and coworkers have previously demonstrated that D-Ins(1,4,6)$P_3$ released $Ca^{2+}$ from intracellular stores. These structure-activity relationships have been discussed in Chapter 4.

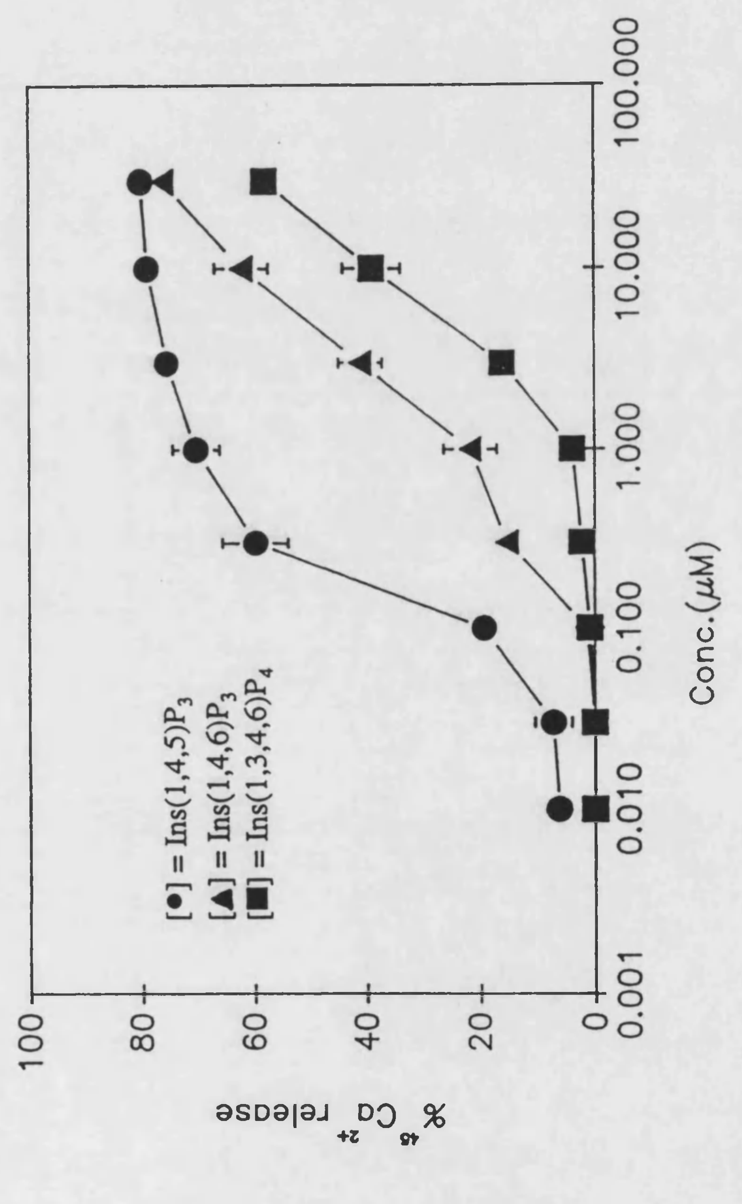
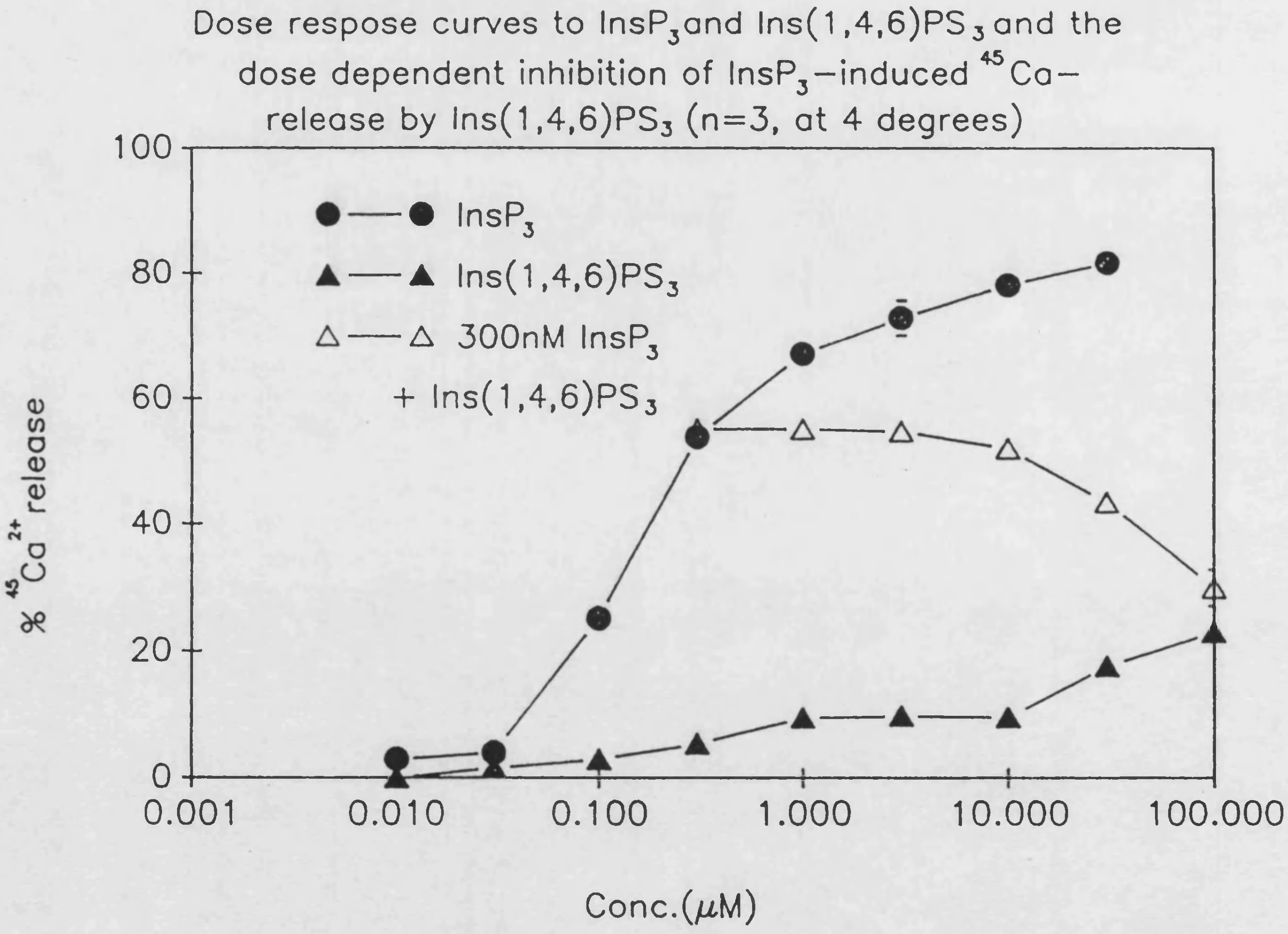



**Figure 103**

For this assay, rabbit platelets were isolated and washed according to Murphy and coworkers. [479] The platelets ($10^9$/ml) were suspended in an "intracellular like" buffer containing 5mM ATP and permeabilised by treatment with saponin (40µg/ml) for 1min. The washed platelets were loaded with $^{45}Ca^{2+}$ for 60min and stimulated with the inositol phosphate analogue for 3min at room temperature. The remaining cell-associated $^{45}Ca^{2+}$ was then determined by rapid filtration. The dose response curves for DL-Ins(1,4,6)$P_3$ are shown in Figure 103. The $EC_{50}$ values (mean ± SEM) were 0.18 ± 0.1µ M for Ins(1,4,5)$P_3$, 2.07 ± 0.08µM for DL-Ins(1,4,6)$P_3$ and 9.67 ± 0.50µM for Ins(1,3,4,6)$P_4$. DL-Ins(1,4,6)$P_3$ **(334)** was some 11-fold less potent than Ins(1,4,5)$P_3$ but 5-fold more potent than Ins(1,3,4,6)$P_4$ in its ability to release $Ca^{2+}$. Only the D-enantiomer of DL-Ins(1,4,6)$P_3$ will release $Ca^{2+}$, as the L-enantiomer does not have a D-4,5-bisphosphate moiety. Thus, D-Ins(1,4,6)$P_3$ **(199)** should only be five to six times less potent at $Ca^{2+}$-release than Ins(1,4,5)$P_3$. [480] DL-Ins(1,4,6)$P_3$ was also evaluated as an inhibitor of 5-phosphatase from HEG and was found to inhibit [$^3$H]Ins(1,4,5)$P_3$ dephosphorylation with a $K_i$ value of 17.8µM.

The $Ca^{2+}$-release activity for DL-Ins(1,4,6)$PS_3$ was evaluated in the same way as for DL-Ins(1,4,6)$P_3$ and the dose response curves are shown in Figure 104. In marked contrast to DL-Ins(1,4,6)$P_3$ the phosphorothioate analogue released a maximum of 21.2% ± 0.40 (n = 3) of the $Ca^{2+}$-released by Ins(1,4,5)$P_3$. For comparison, L-*chiro*-Ins(2,3,5)$PS_3$ and *scyllo*-Ins(1,2,4,5)$PS_4$ released 42.3 ± 6% (n = 3) and 61.7 ± 11% (n = 3) respectively. Of the three phosphorothioate analogues described above, only DL-Ins(1,4,6)$PS_3$ exhibited antagonism at the Ins(1,4,5)$P_3$ receptor. Coincubation of DL-Ins(1,4,6)$PS_3$ (300nM-100µM) with 0.3µM of Ins(1,4,5)$P_3$ at 0°C produced a dose dependent inhibition of Ins(1,4,5)$P_3$-induced $^{45}Ca^{2+}$-release, such that 100µM of DL-Ins(1,4,6)$PS_3$ produced approximately 50% inhibition, (in Figure 104). This analogue was the first description of an antagonist at the Ins(1,4,5)$P_3$ receptor in platelets. This molecule possesses the best antagonistic properties so far described, which, by further modification will hopefully lead to a full antagonist. The precursors to the chiral D-enantiomer have been resolved and pure D-Ins(1,4,6)$PS_3$ has been synthesised, but the pharmacology on this compound has not been carried out. J. Al-Hafidh, carried out the biological testing of DL-Ins(1,4,6)$P_3$ and DL-Ins(1,4,6)$PS_3$ at the University of Bath.



Dose respose curves to InsP3 and Ins(1,4,6)PS3 and the dose dependent inhibition of InsP3−induced 45Ca−release by Ins(1,4,6)PS3 (n=3, at 4 degrees)
InsP3
Ins(1,4,6)PS3
300nM InsP3
+ Ins(1,4,6)PS3
% 45Ca2+ release
Conc.(μM)
0
20
40
60
80
100
0.001
0.010
0.100
1.000
10.000
100.000


Figure 104

## 5.6 Resolution of DL-1,2,5-Tri-*O*-Benzyl-*myo*-Inositol Precursors

From the previous section DL-1,4-di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(330)** was synthesised from DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol and the *cis*-2,3-diol was resolved successfully with (*S*)-(+)-*O*-acetylmandelic acid to provide the enantiomers of 1,2,5-tri-*O*-benzyl-*myo*-inositol. This was carried out in the following manner.

A mixture of DL-1,4-di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(330)**, (*S*)-(+)-*O*-acetylmandelic acid **(328)** (1.04 equivalents) and a catalytic amount of DMAP in dichloromethane was stirred at -20°C, (in Figure 105). DCC in dichloromethane was added dropwise over a period of 1.5h, the cooling was removed and the mixture was stirred overnight at room temperature. TLC (pentane-ethyl acetate, 2:1) showed two products, $R_f$ = 0.28 and 0.40. The precipitate of DCU was removed by filtration through celite and the clear solvent was evaporated to give a syrup. The mixture was purified by flash chromatography to give the two diastereoisomers. No problems were encountered in the purification process, in which there was little overlap during chromatography. Of the two diastereoisomers, D-1-*O*-[*S*-(+)-*O*-acetylmandelyl]-3,6-di-*O*-allyl-5-*O*-benzyl-4-*O*-*p*-methoxybenzyl-*myo*-inositol **(337)**, was isolated as the less polar isomer ($R_f$ = 0.40) in 36.5% yield as a syrup $[\alpha]_D$ = -8°, and L-1-*O*-[*S*-(+)-*O*-acetylmandelyl]-3,6-di-*O*-allyl-5-*O*-benzyl-4-*O*-*p*-methoxybenzyl-*myo*-inositol **(338)**, as the more polar, ($R_f$ = 0.28) [isolated as a crystalline solid in 35% yield, m.p. 103-105°C, (from ethanol), and $[\alpha]_D$ = +59°]. A single X-ray crystal structure was attempted, however, the shape of the unit crystal and weak diffraction patterns made it difficult for a successful crystal structure determination. Establishing the absolute configuration of the two diastereoisomers will be discussed later.

The $^1$H NMR spectra for the two diastereoisomers is shown in Figure 106. The upper spectrum represents compound **(337)** and the lower spectrum compound **(338)**. Unlike the resolution of the tetrabenzyl derivative used for the chiral synthesis of D- and L-Ins(1,2,4,5)$P_4$, there were fewer distinct differences between the signals of the diastereoisomers in the aromatic region and the unique hydrogen (as a singlet) of the acetylmandelate moiety $CH_3CO_2CH$(Ph)$CO_2$- overlapped with the allyl hydrogen Ins-O-$CH_2CH=CH_2$ signal in the spectra. A closer look at the upper spectrum revealed two allyl groups overlapping (at ~ $\delta$ = 5.9) (Ins-O-$CH_2CH=CH_2$) which is the usual shift for this functional group. However, for the more polar diastereoisomer the multiplet at $\delta$ = 5.91 only integrated for one hydrogen. The other hydrogen from Ins-O-$CH_2CH=CH_2$

and presumably attached at the C-6 position was shifted upfield to $\delta = 5.30$, presumably due to the shielding affect of the acetylmandelate phenyl moiety.

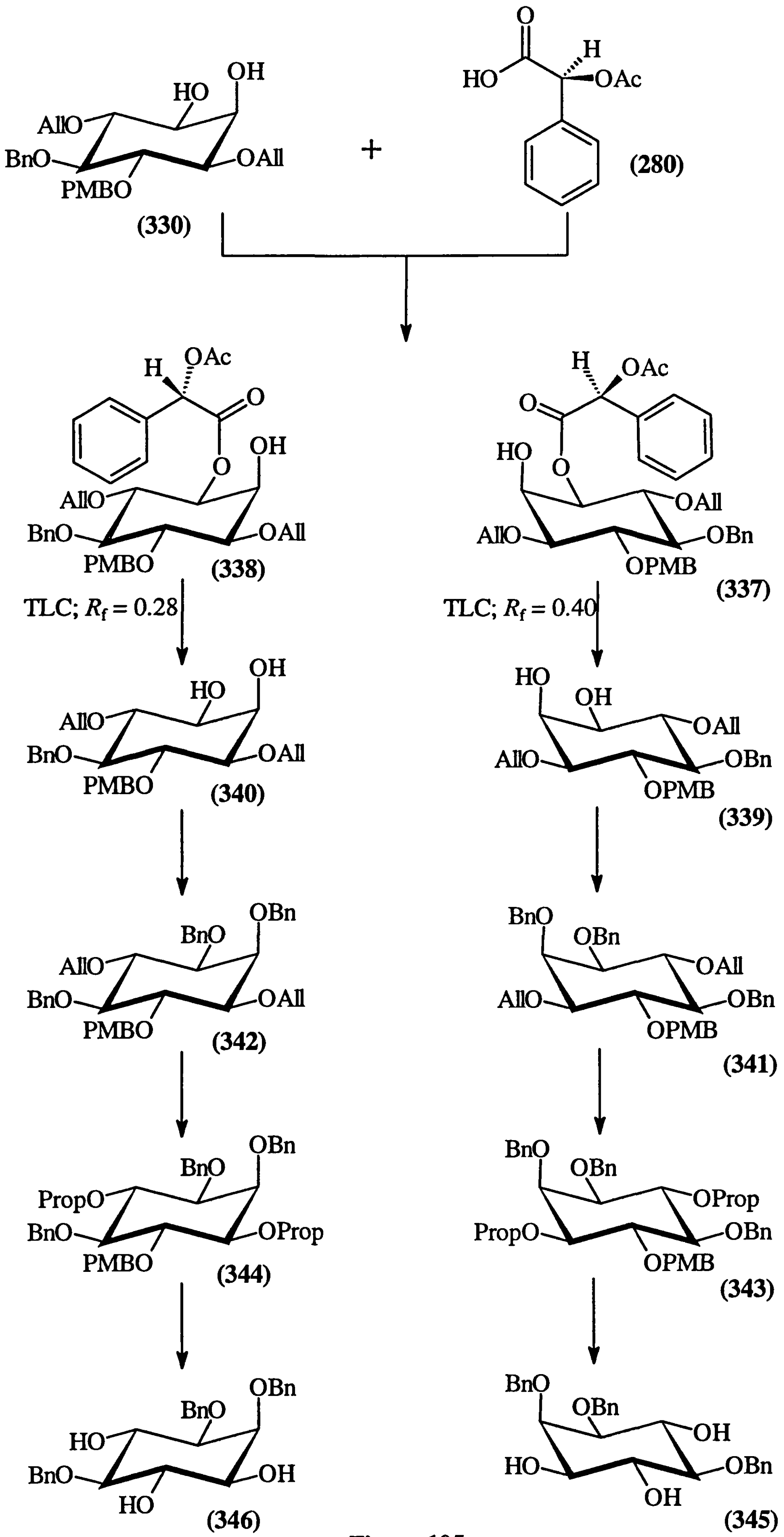

(330)
(280)
(338)
(337)
TLC; $R_f$ = 0.28
TLC; $R_f$ = 0.40
(340)
(339)
(342)
(341)
(344)
(343)
(346)
(345)

**Figure 105**

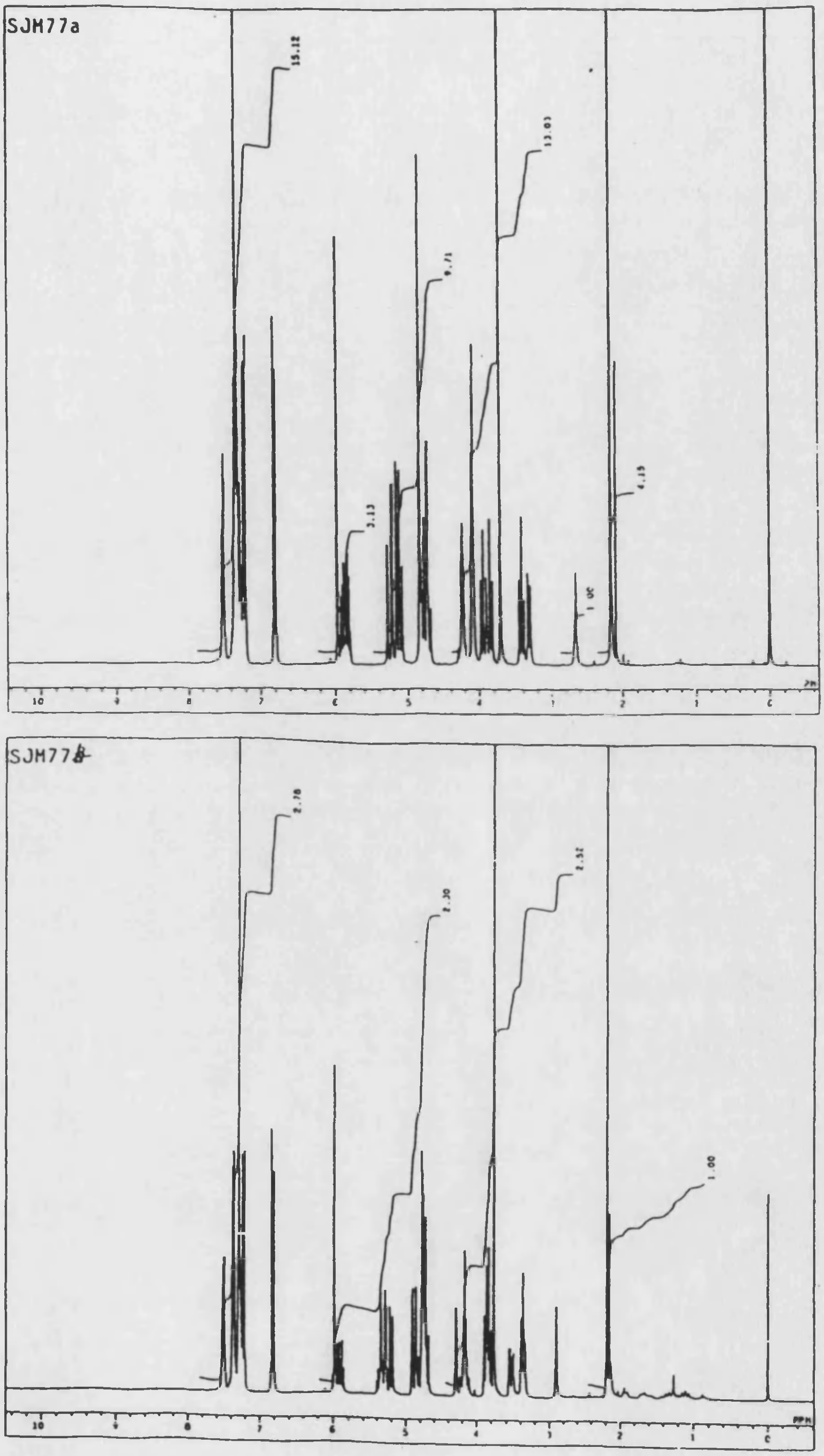
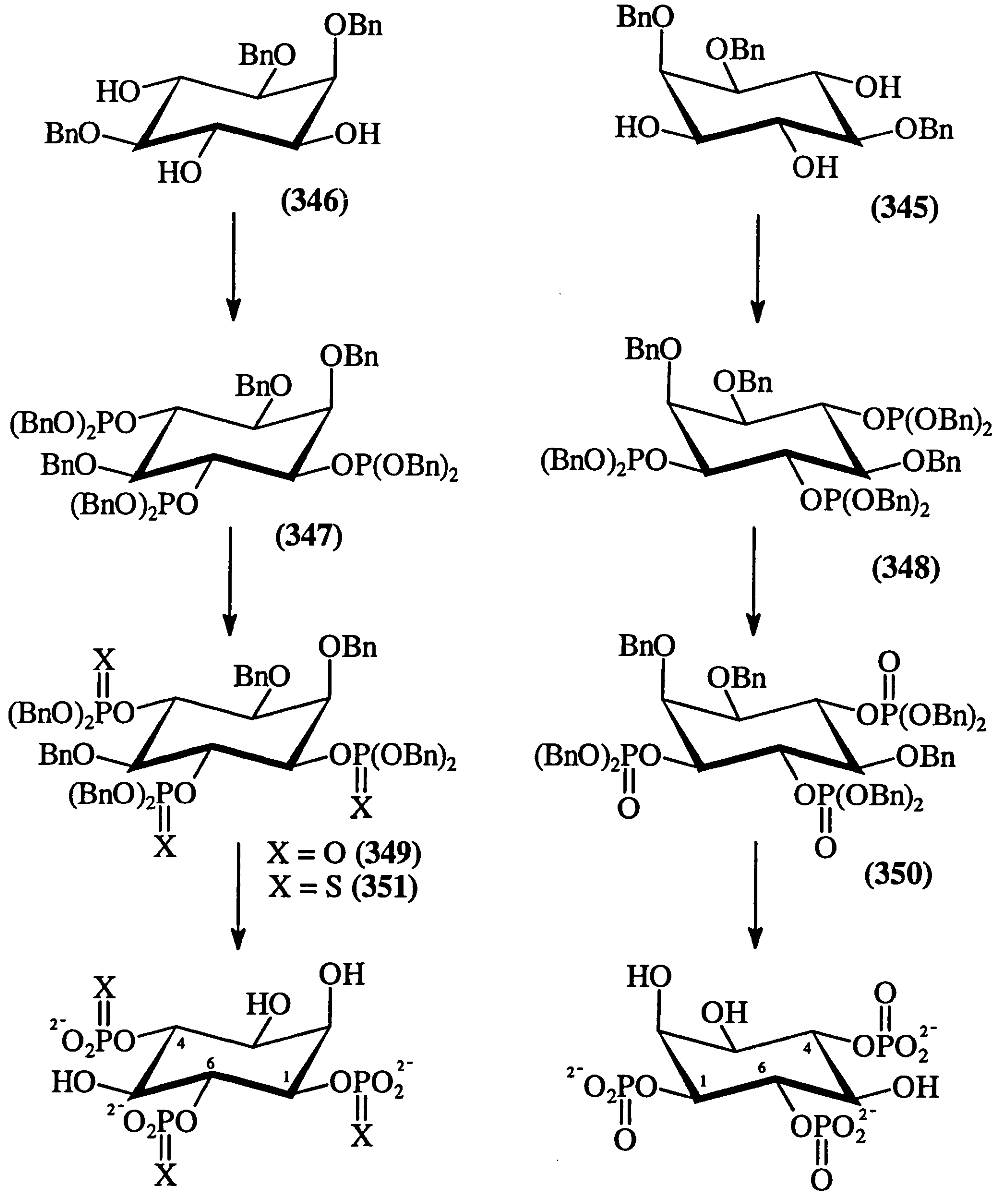



**Figure 106**

C-1-H of the less polar diastereoisomer has been shifted downfield, due to the carbonyl deshielding effect of the acetylmandelate, which was be identified as a dd, $\delta = 4.77$, J = 2.56 and 10.26Hz. Thus selective acylation occurred at the 1-position. The dd, for the slower running compound by TLC was not be seen, however, C-2-H of the more polar diastereoisomer **(338)** showed a broad singlet at $\delta = 4.29$, thus C-1-OH was selectively acylated in both diastereoisomers. The hydroxyl group at C-2 for compound **(338)** $\delta = 2.65$ is more shielded than for the slower moving diastereoisomer at $\delta = 2.90$. The $^{13}C$ NMR spectra did not show any significant differences in chemical shift values between similar groups.

The individual diastereoisomers were then deacylated with methanolic sodium hydroxide solution to give L-1,4-di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(339)**, (from the faster moving diastereoisomer) in 90% yield, after work up and crystallisation from ethyl acetate-hexane. The specific rotation was $[\alpha]_D = -51°$, and the melting point (111-113°C) was 24°C higher than for the racemic material. The D-enantiomer, D-1,4-di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(340)**, was obtained in the same way, and isolated in 98% yield. The specific rotation was equal and opposite (+51°) and the melting point was found to be the same (111-112°C). The hydroxyl groups at the *cis* 2,3-diol of each enantiomer were benzylated using benzyl bromide and sodium hydride in DMF, to give the fully protected compounds **(341)** and **(342)** in 78% and 94% yields respectively. The melting point for both enantiomers was 72-73°C and 19°C higher than for the racemic derivative. Both specific rotations were equal and opposite such that compound **(341)** was $[\alpha]_D = -19°$ and compound **(342)** $[\alpha]_D = +19°$. The allyl and *p*-methoxybenzyl groups were cleaved in a two step process for each enantiomer. First, the two allyl groups of each compound were isomerised using freshly sublimed potassium *t*-butoxide in anhydrous DMSO for 5h at 50°C. TLC (ether-hexane, 1:1) showed complete conversion of starting material $R_f = 0.50$ into a single product $R_f = 0.72$, indicating the formation of the 1,4-di-*O*-*cis*-prop-1-enyl derivative **(343,344)** which were not isolated. The individual enantiomers were extracted and the acid sensitive *cis*-prop-1-enyl and *p*-methoxybenzyl groups were cleaved under acid conditions using 1M aqueous HCl in ethanol (1:2) at reflux temperature for 3h. The solvents were evaporated *in vacuo* and the resulting solids were purified by flash chromatography to give L-2,3,5-tri-*O*-benzyl-*myo*-inositol **(345)**, used for the synthesis of L-Ins(1,4,6)$P_3$ and D-2,3,5-tri-*O*-benzyl-*myo*-inositol **(346)**, used for the synthesis of D-Ins(1,4,6)$P_3$. The two triols were isolated in 67% yield for **(345)** and in 71% yield for **(346)**. The melting point (176-177°C) for both enantiomers was also higher than the racemate by 16°C and the specific rotations $[\alpha]_D = -34°$ and $[\alpha]_D = +34°$ were equal and opposite. The NMR data were the same as for the racemic material.

### 5.6.1 Synthesis of D- and L-Ins(1,4,6)$P_3$




D-Ins(1,4,6)$P_3$ X = O **(199)**
D-Ins(1,4,6)$PS_3$ X = S **(352)**

L-Ins(1,4,6)$P_3$ **(201)**

**Figure 107**

The chiral triols were essentially synthesised in the same way as for the racemic mixture and summarised in Figure 107. The difference was in the chromatography solvent which was changed to chloroform-acetone (10:1). It was found that this system easily separated the phosphonate impurities from the required totally protected product. The specific rotations also provided a problem because both compounds appeared to read zero on the polarimeter. Thus the readings have been reported as 0° ± 1° for each of the totally protected compounds **(349)** and **(350)**. This specific rotation was then carried out at other wavelengths and the same result was found.

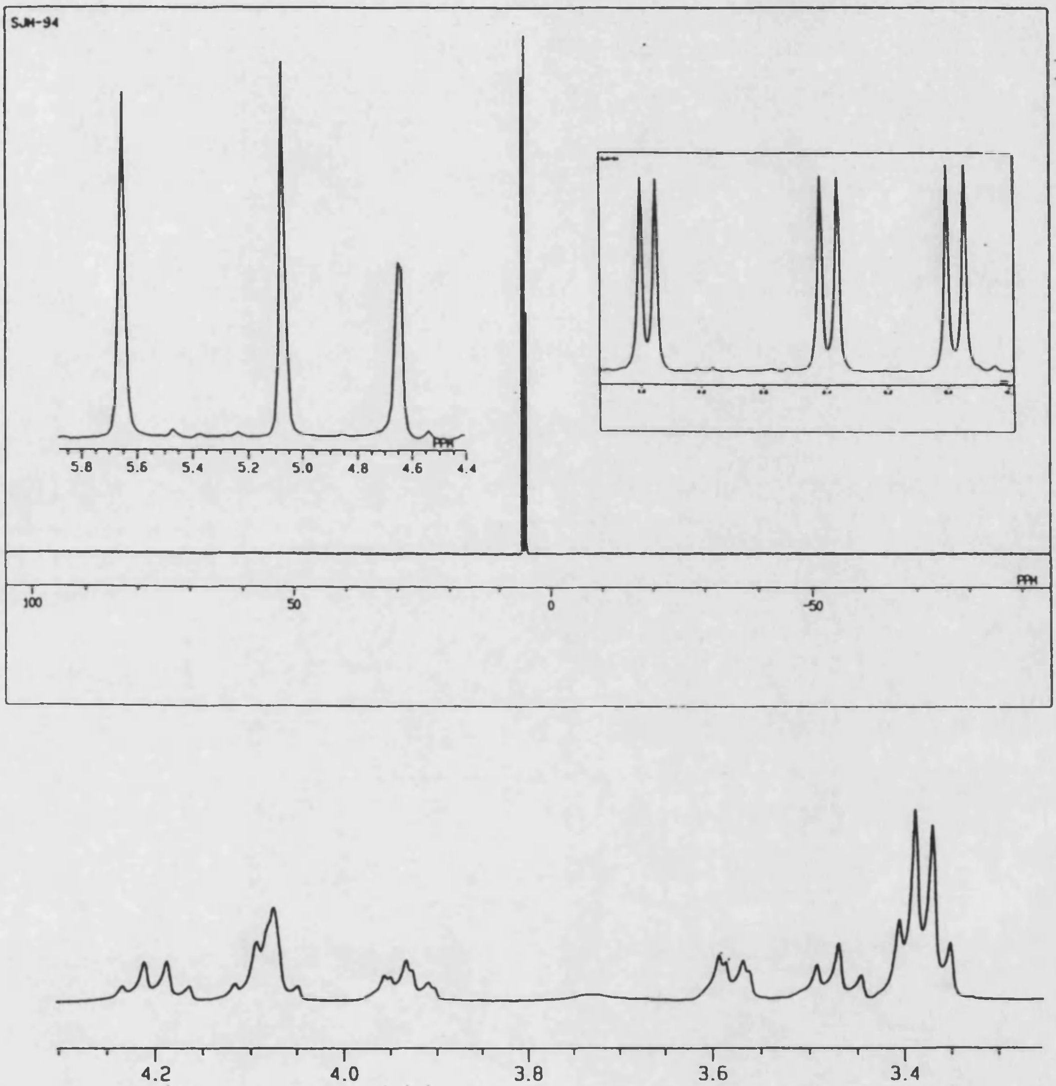



**Figure 108**

Deprotection of the enantiomers with sodium in liquid ammonia followed by purification on Q-Sepharose Fast Flow using a gradient of TEAB as buffer, gave D-Ins(1,4,6)$P_3$ **(199)** in 34% yield and L-Ins(1,4,6)$P_3$ **(201)** in 60% yield. The rotations for the respective compounds at pH 8.6 in 1M TEAB buffer were $[\alpha]_D$ = -29.1° and $[\alpha]_D$ = +25.0°. The size of the rotation in TEAB buffer was much larger than in water, probably due to the interaction of the triethylamine moiety with the phosphate groups and was also observed for the tetrakisphosphates. The literature values, which have just been published, for D-Ins(1,4,6)$P_3$ and L-Ins(1,4,6)$P_3$ were $[\alpha]_D$ = -8.9° (c = 0.90, $H_2O$) and $[\alpha]_D$ = +9.4° (c = 0.85, $H_2O$), prepared by a totally different route by Ozaki and coworkers. Thus the sign of the rotation of each enantiomer **(199,201)** agreed with that prepared by Ozaki and coworkers. [432]

The $^1H$ and $^{31}P$ NMR spectra for D-Ins(1,4,6)$P_3$ are given in Figure 108. For the $^1H$ NMR spectrum at $\delta$ = 3.44, (t, J = 9.15Hz for C-5-H) a $\delta$ = 3.58, (dd, J = 9.77 and 2.5Hz, for C-3-H), whilst C-1-H was at $\delta$ = 3.93, (br dt, J = 9.46 and 2.5Hz). The two overlapping signals at $\delta$ = 4.07 were C-2-H as a broad singlet and C-4-H (q, J = 9.16Hz). C-6-H was deshielded more than C-4-H and appeared at $\delta$ = 4.20 (q, J = 9.16Hz). The $^{31}P$ NMR (162MHz) showed three singlets in the $^{31}P$-$^1H$-decoupled spectrum and as doublets in the $^1H$-$^{31}P$-coupled spectrum, $\delta$ = -0.03 (d, J = 9.52Hz), $\delta$ = +0.39 (d, J = 8.79Hz) and $\delta$ = +0.97 (d, J = 8.3Hz). Both compounds gave satisfactory accurate -ve FAB spectra.

### 5.6.2 Synthesis of D-Ins(1,4,6)$PS_3$

The phosphitylating procedure was carried out as for the racemic compound, and the route is shown in Figure 107. Sulphoxidation was carried out in the same way as for the racemic compound in order to obtain the totally protected D-trisphosphorothioate **(351)**. The work up and purification steps for the protected intermediate were also the same as for the racemic compound. The specific rotation was low because the polarimeter indicated a zero reading as for the protected chiral phosphates, thus a rotation of 0° ± 1° was registered at 589nm and at other wavelengths. Deprotection of the fully blocked compound using sodium in liquid ammonia followed by purification on Q-Sepharose, eluting with a gradient of TEAB buffer gave D-Ins(1,4,6)$PS_3$ **(352)** in 36.5% yield. The specific rotation for D-Ins(1,4,6)$PS_3$, was found to be -26.9° at pH 8.6 in TEAB buffer and was similar to D-Ins(1,4,6)$P_3$.

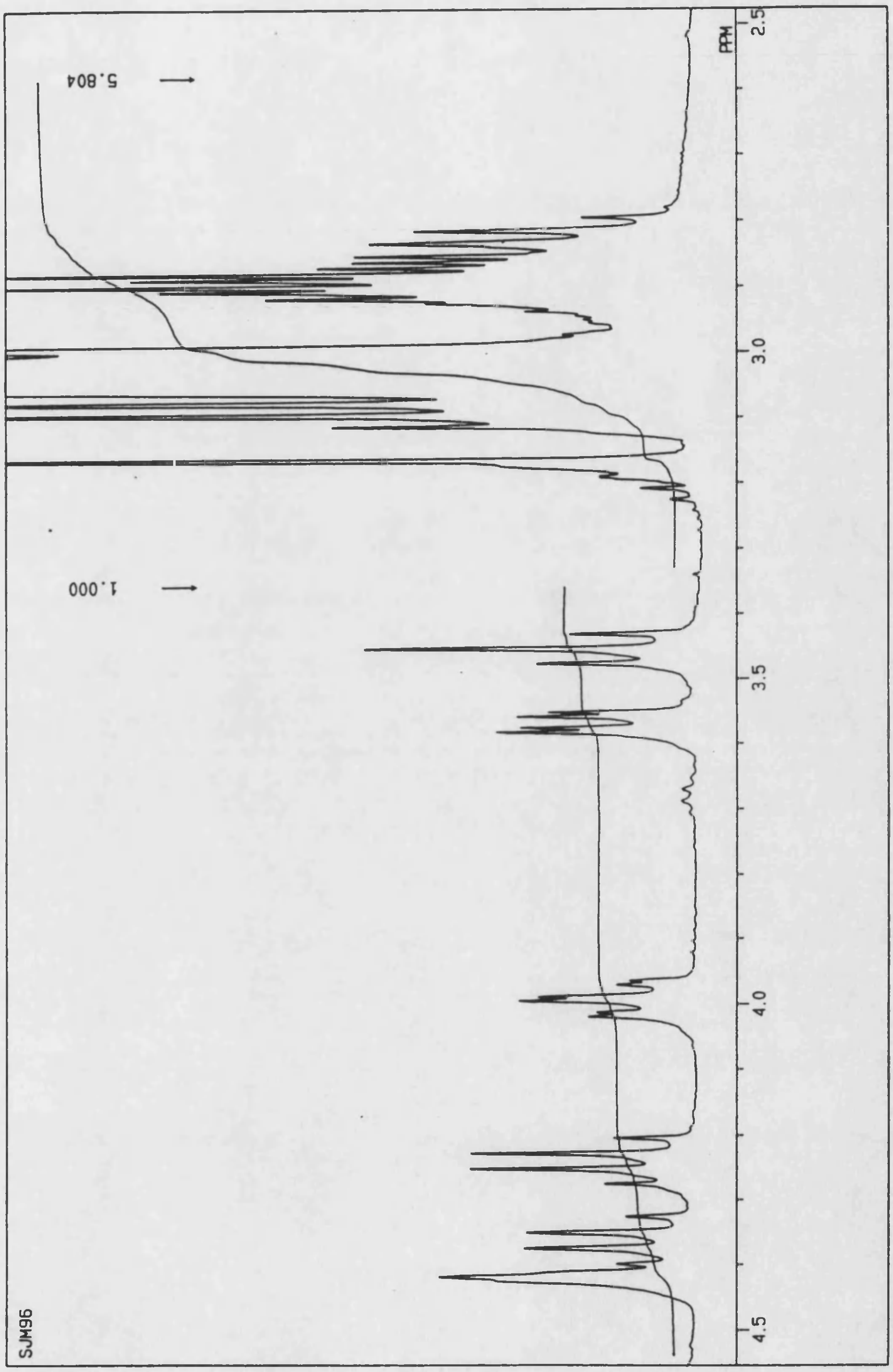


Figure 109

The $^{1}H$ NMR spectrum for D-Ins(1,4,6)$PS_3$ is given in Figure 109 and was the best recorded spectrum for a water soluble *myo*-inositol phosphate or phosphorothioate analogue prepared by the author. The spectrum shows six clear signals at $\delta = 3.42$ (t, J = 9.16Hz for C-5-H) and $\delta = 3.56$ (dd, J = 2.75, 9.77Hz for C-3-H) and both these positions were unphosphorylated. The next three signals were the phosphorylated hydroxyl positions at $\delta = 3.99$ (dt, J = 2.74, 9.77Hz for C-1-H), $\delta = 4.24$ (q, J = 9.46Hz for C-4-H) and $\delta = 4.36$ (q, J = 9.76Hz for C-6-H) and C-2-H was seen at $\delta = 4.42$ as a singlet. The $^{31}P$ NMR spectra for D-Ins(1,4,6)$PS_3$ are given in Figure 110. The $^{31}P$ spectrum showed three sharp singlets and as doublets in the $^{1}H$-$^{31}P$ coupled spectrum, at $\delta = +47.31$ (d, J = 11.45Hz), and $\delta = +48.24$ (d, J = 11.44Hz) and $\delta = +50.00$ (d, J = 9.92Hz). This compound also gave a satisfactory accurate -ve FAB.

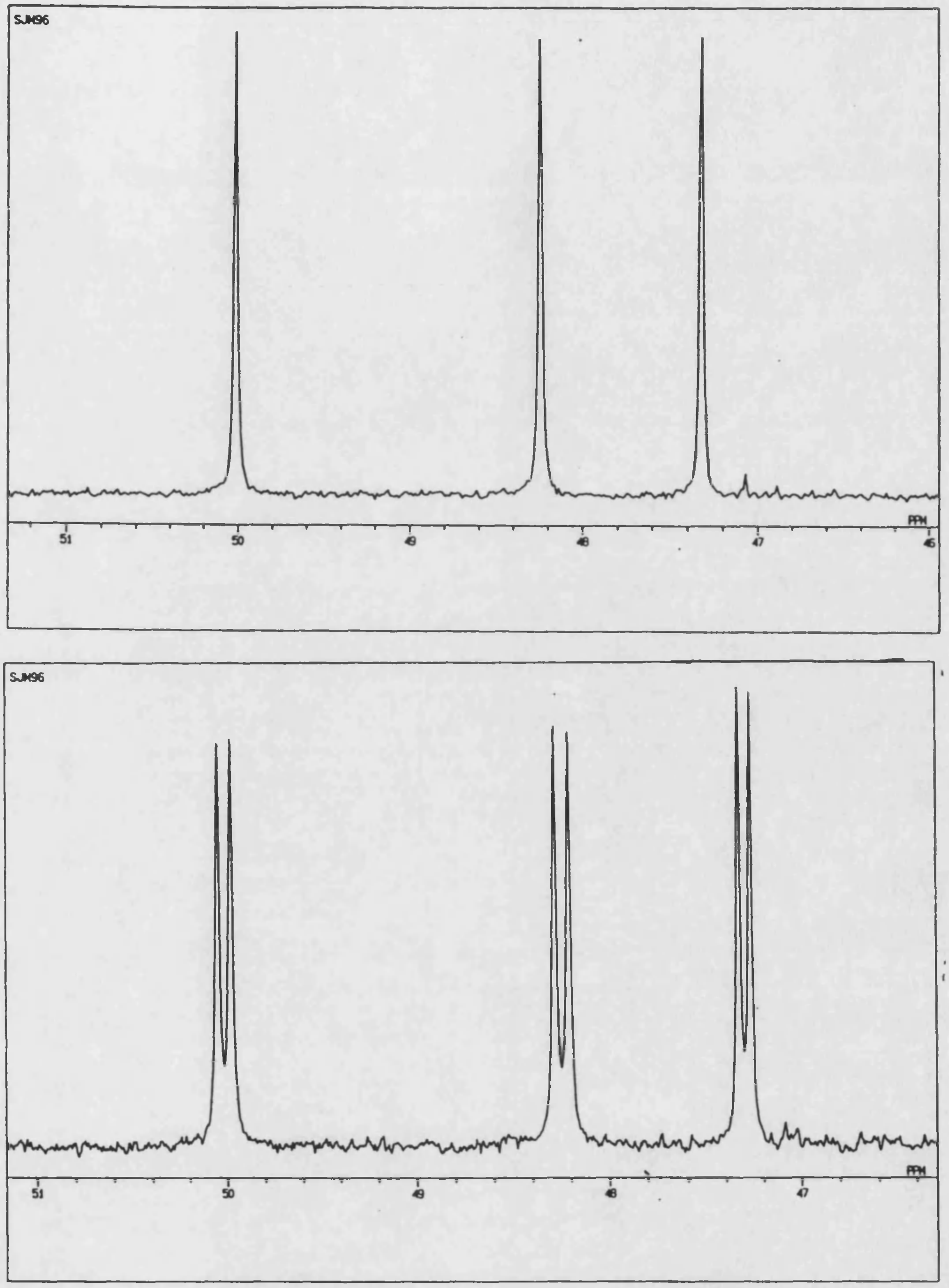



**Figure 110**

### 5.6.3 Establishing the Absolute Configuration D-2,3,5-Tri-*O*-Benzyl-*myo*-Inositol

A literature search was used to find a possible target compound that could be synthesised in a small number of steps in order to identify the absolute configuration of D-2,3,5-tri-*O*-benzyl-*myo*-inositol **(346)**. From the literature, we found D- and L-1,2,4,5-tetra-*O*-benzyl-*myo*-inositol whose rotations were $[\alpha]_D$ = -4.3° and +3.9° respectively, which had a melting point of 105-107°C. [386] Gigg and coworkers determined the absolute configuration of these two compounds from the known derivatives D- and L-2,4,5-tri-*O*-benzyl-*myo*-inositol, by selective benzylation at the 1-position to give the respective D- and L-1,2,4,5-tetra-*O*-benzyl-*myo*-inositol derivatives.

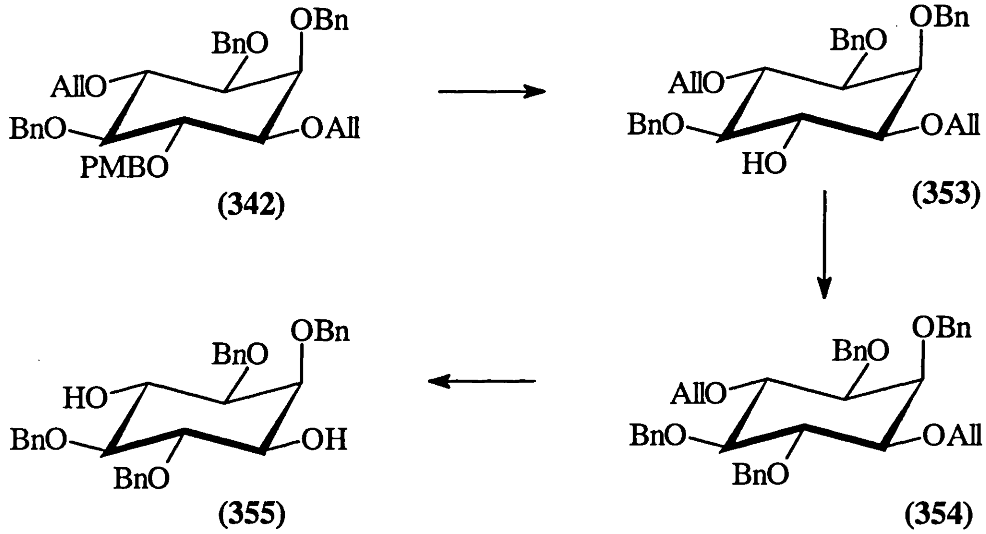



**Figure 111**

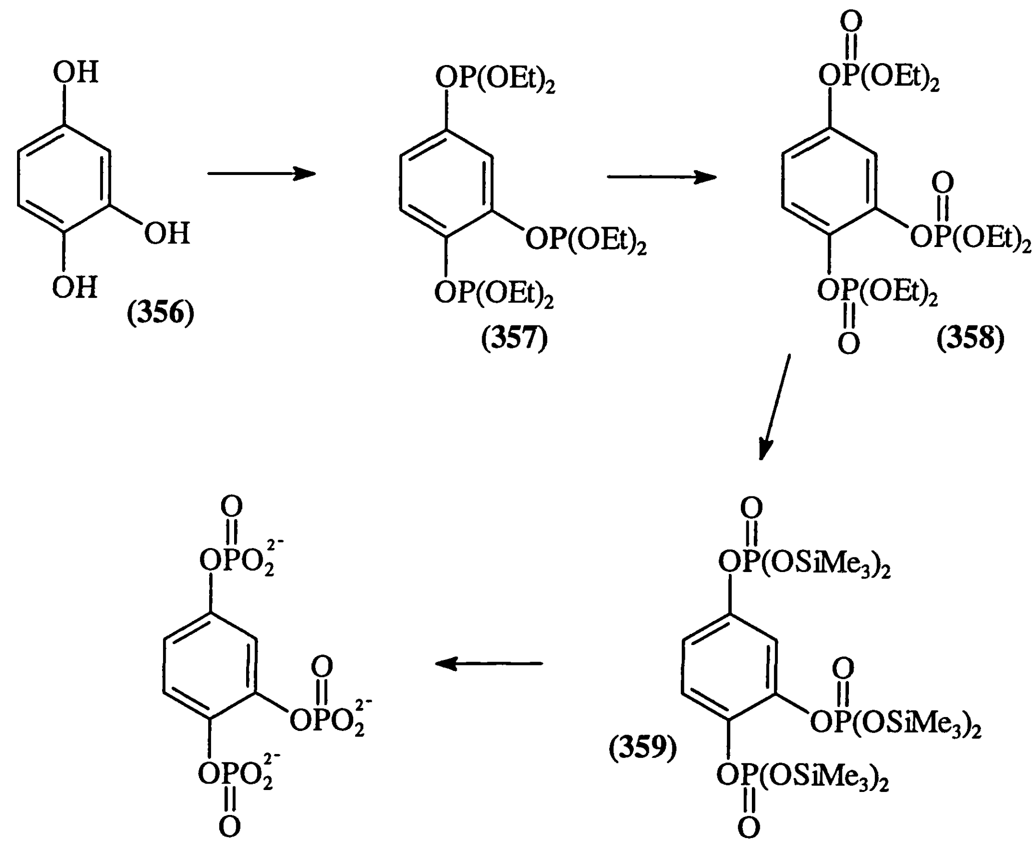
L-1,2,4,5-Tetra-*O*-benzyl-*myo*-inositol (**355** in Figure 111) was synthesised from D-1,4-di-*O*-allyl-2,3,5-tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(342)**. The *p*-methoxybenzyl group was removed from **(342)** by acidic hydrolysis using 1M HCl-ethanol (1:2) at reflux temperature for 4h. The solvents were evaporated *in vacuo* followed by work up and purification by flash chromatography to provide D-1,4-di-*O*-allyl-2,3,5-tri-*O*-benzyl-*myo*-inositol **(353)** in 91% yield as a syrup, $[\alpha]_D$ = -3° (c = 9 in $CH_2Cl_2$). The exposed 6-hydroxyl group was then benzylated with benzyl bromide and sodium hydride in DMF to give D-1,4-di-*O*-allyl-2,3,5,6-tetra-*O*-benzyl-*myo*-inositol **(354)** in 90% yield after work up and flash chromatography. Gigg and coworkers [386] prepared racemic **(354)** which was isolated as a syrup, however, chiral **(354)** was a low melting point solid, 63-65°C (from pentane and had a rotation of $[\alpha]_D$ = +18° (c = 1 in $CH_2Cl_2$). The allyl groups were then removed in a one pot reaction using palladium on carbon in the presence of toluene-*p*-sulphonic acid at reflux temperature for 2h in ethanol-water (6:1, 30ml). First the allyl group was isomerised to a mixture of the *cis* and *trans*-prop-1-enyl deivatives, and second the prop-1-enyl derivatives were hydrolysed with toluene-*p*-sulphonic acid. The mixture was filtered through celite and the solvents were evaporated. The residue was then purified by flash chromatography (ethyl acetate-dichloromethane, 1:1) to give pure D-2,3,5,6-tetra-*O*-benzyl-*myo*-inositol **(355)** in 61% yield, which is the same as L-1,2,4,5-tetra-*O*-benzyl-*myo*-inositol. The specific rotation was found to be $[\alpha]_D = +4.0°$ (c = 2 in $CH_2Cl_2$), and agreed well with the literature value [386] of $[\alpha]_D = +3.9°$ (c = 1 in $CHCl_3$). The melting point of the compound was also similar, (103-105°C) and in good agreement with the literature value of, 105-107°C.

After this work had been completed, the synthesis of D- and L-Ins(1,4,6)$P_3$ by a different route was published and the sign of the rotational values were the same for each enantiomer, although the size of the rotation was different due to the nature of the counterions and solvent used for this determination.

### 5.7 Synthesis of Benzene 1,2,4-Trisphosphate

An analogue loosely based on the arrangement of the phosphate groups at the 1-, 4- and 5-positions was benzene 1,2,4-trisphosphate **(360)**. In this analogue, the 4-, 1- and 2-positions represent the 1-, 4- and 5-positions of Ins(1,4,5)$P_3$ respectively. However, there are no hydroxyl groups at the 3-, 5- and 6-positions of the benzene ring.

The synthesis of benzene 1,2,4-trisphosphate (**360** in Figure 112) was carried out in a few steps using commercially available starting materials. Benzene 1,2,4-triol **(356)** was obtained from Aldrich Chemical Company and was in the form of an amorphous grey powder. When the triol **(356)** was added to a mixture of dry *N,N*-diisopropylethylamine in dry dichloromethane, the solution changed immediately to a blood red colour. The solution was then cooled to -78°C with a dry ice-acetone cooling bath. Diethoxychlorophosphine was then added dropwise and the hydroxyl groups were phosphitylated when the solution changed from blood red to a pale yellow colour. At the 1,2,4-trisphosphite triester stage **(357)** an AB $^{31}P$-$^{31}P$ coupling pattern was observed for the 1- and 2-positions, $J_{1,2}$ = 6.10Hz, centered around $\delta$ = +134ppm and $\delta$ = +133.2ppm for the 4-position. The cooling was then removed and the excess phosphitylating reagent was hydrolysed with water (2ml) after which the trisphosphite **(357)** was oxidised to the trisphosphate **(358)** with *t*-butylhydroperoxide and the AB coupling pattern was destroyed. After work up and flash chromatography the pure trisphosphate triester was isolated as a syrup in 76% yield $R_f$ = 0.34 (ethyl acetate-ethanol, 9:1).



Benzene 1,2,4-trisphosphate (360)

**Figure 112**

The six ethyl groups of 1,2,4-tris(diethoxyphospho)-benzene **(358)** were removed in a two step process. First, an excess of bromotrimethylsilane was added to a solution of **(358)** in dry dichloromethane. The reaction was stirred overnight, after which the protective ethyl groups had been replaced with trimethylsilyl moieties, in quantitative yield as judged by $^{31}P$ NMR. The three phosphorus peaks for **(358)** at $\delta$ = -3.61, -3.92 and -4.28ppm were shifted upfield to $\delta$ = -22.14, -22.61 and -23.35ppm for **(359)**. The lower boiling solvents were evaporated and the silicon protective groups were then removed from **(359)** by the addition of water (1ml).

Final purification of the product was achieved by elution from Q-Sepharose Fast Flow using a linear gradient of TEAB buffer, 0-1000mmol. Benzene 1,2,4-trisphosphate **(360)** was eluted between 200-500mM buffer and was evaporated to give a glassy triethylammonium salt in 86% yield. The Briggs phosphate test was only used for the quantitative test because **(360)** was detected using the UV monitor. The three signals in the $^{31}P$ NMR spectrum of (**360** in Figure 113) showed three singlets at -3.61, -3.92 and -4.28ppm. A satisfactory accurate -ve FAB mass spectrum was also obtained for **(360)**.

Attempts were made to synthesise benzene 1,2,4-trisphosphorothioate. However, when sulphoxidation of the trisphosphite was carried out using sulphur in pyridine-DMF, the only product that formed judged by $^{31}P$ NMR was the symmetrical thiopyrophosphate at $\delta = +56$ppm. This compound will be synthesised later by a different approach using another sulphurising reagent.

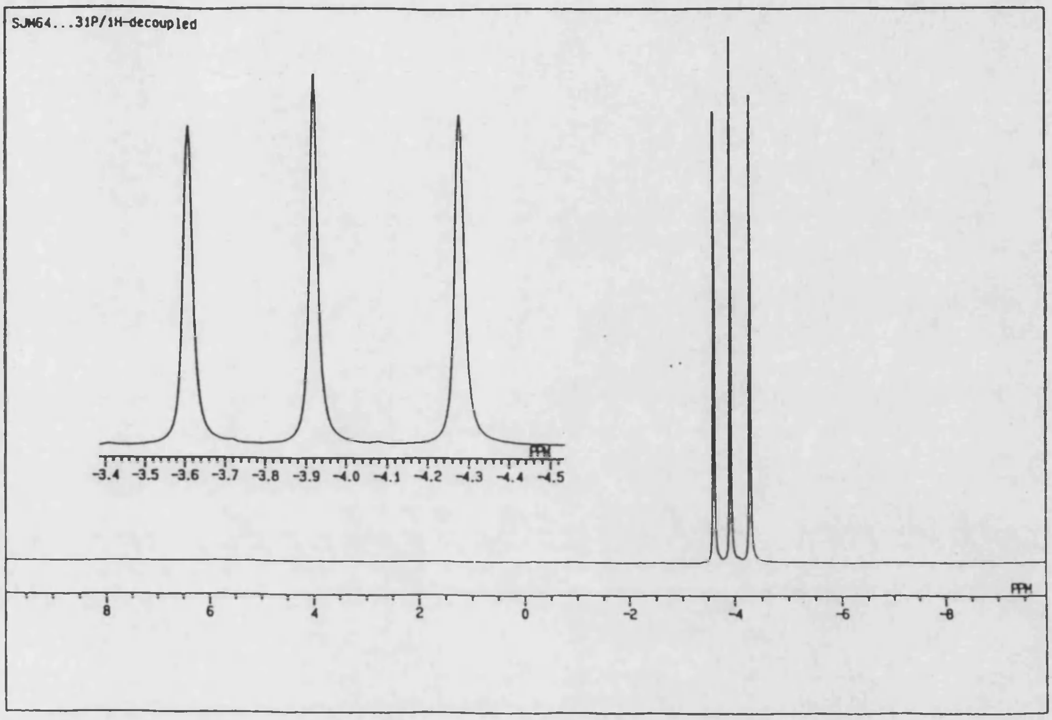



**Figure 113**

### 5.7.1 Pharmacology

Benzene 1,2,4-trisphosphate **(360)** has been prepared by another group **[461]** and by a different method using P(V) phosphorylation and the phosphorylated intermediate was not purified or characterised. It was found by these workers that this compound inhibited 3-kinase phosphorylation of $[^3H]Ins(1,4,5)P_3$ with an $IC_{50}$ = 6.1μM (from bovine adrenal cortex). It also inhibited the dephosphorylation of $[^3H]Ins(1,4,5)P_3$ with an $IC_{50}$ = 32μM (from adrenal cortex microsomes) which indicated in this system at least, the affinity of **(360)** for $Ins(1,4,5)P_3$ phosphatase was similar to or slightly higher than $Ins(1,4,5)P_3$. This compound was also found to be a very weak antagonist at the

Ins(1,4,5)$P_3$ receptor, $IC_{50}$ = 428μM from a microsomal preparation. Thus, further modification of this structure may lead to a full antagonist.

The benzene 1,2,4-trisphosphate we prepared was tested on 5-phosphatase from HEG preparations, and had a $K_i$ value of 7μM, (carried out by S. T. Safrany at Leicester University). No data on 3-kinase activity were available. However, this compound was tested on PtdIns 3-kinase, an enzyme which phosphorylates the minor phospholipid PtdIns(4,5)$P_2$ to give PtdIns(3,4,5)$P_3$. It was found (by S. Ward at Bath University), that **(360)** inhibited PtdIns 3-kinase activity in p85 mAb (monoclonal antibody) immunoprecipitates with an $IC_{50}$ value of 25μM ± 2.5. It was also found that L-*chiro*-Ins(2,3,5)$P_3$ and L-*chiro*-Ins(2,3,5)$PS_3$ discussed earlier also inhibited the PtdIns 3-kinase activity in p85 mAb immunoprecipitates with an $IC_{50}$ value of 5μM ± 3.5 and 20 μM ± 5. The results for these experiments are shown in Figure 114. This is the first report of any inositol phosphate analogues which inhibit PtdIns 3-kinase.

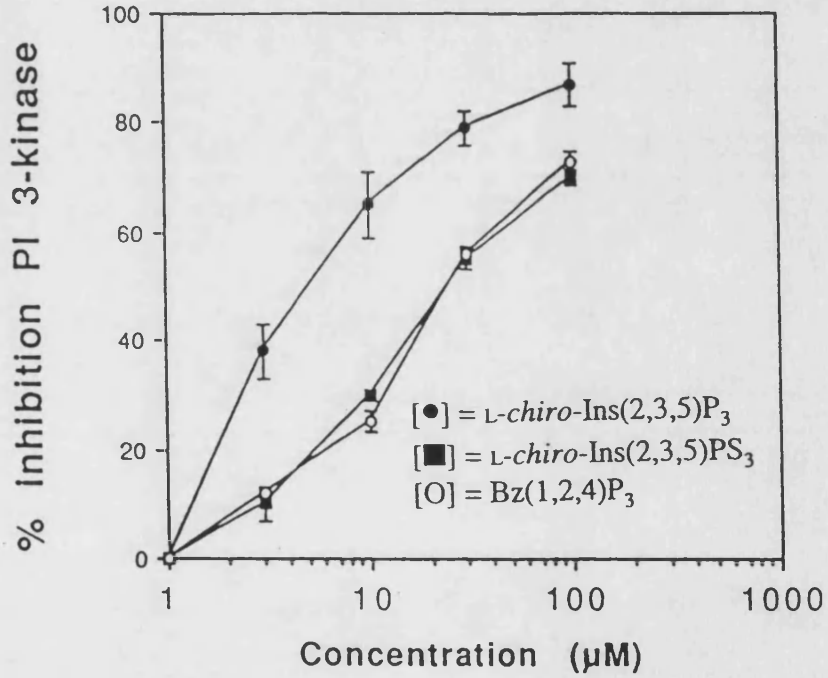



**Figure 114**

### 5.8 Outlook

In 1883 Ringer [176] discovered that cardiac muscle could not continue to beat *in vitro* if a minimum concentration of extracellular $Ca^{2+}$ was absent. Exactly a century later a paper presented in *Nature* [24] described the linkage between the binding of the water soluble molecule Ins(1,4,5)$P_3$ and the release of $Ca^{2+}$ from intracellular stores. Since 1983, the role of Ins(1,4,5)$P_3$ and its metabolism has asked more questions than has given answers, but has also provided new avenues for research, although its importance as a mobiliser of $Ca^{2+}$ ions should not be undermined. The response of the receptor to Ins(1,4,5)$P_3$ in different cell types, in terms of receptor binding and $Ca^{2+}$-release may provide new targets for drug design, once the inherent hydrophilic properties of Ins(1,4,5)$P_3$ analogues have been overcome and new lipophilic molecules have been synthesised.

Specific small molecule antagonists for certain cell types, would be invaluable as tools for investigating the response downstream of the receptor, following the consequences of zero $Ca^{2+}$-release *in vivo*, and may give some indication of specific function for that receptor-type. Analogues that have been described in this thesis, in particular DL-Ins(1,4,6)$PS_3$, have contributed to the development of small molecule antagonists, although this molecule is a partial agonist. Further modification will be necessary to provide a full antagonist. The development of specific and potent small molecule 3-kinase and 5-phosphatase inhibitors would be invaluable, together with inhibitors of enzymes further downstream of Ins(1,4,5)$P_3$. Once an inhibitor has been synthesised, it must pass across the cell membrane in order to reach its target, and cell permeable analogues are another major area for development. [481,482]

# CHAPTER SIX

# Experimental

## 6 General Information For Experimental

Unless stated, *myo*-inositol derivatives were racemic and are drawn in the D-configuration. The naming of the D- and L-enantiomers were not interchanged so that the experimental is easy to follow. Thin layer chromatography (TLC) was performed on pre-coated plates (Merck TLC aluminium sheets silica 60 $F_{254}$): the product was visualised by spraying with phosphomolybdic acid in methanol, followed by heating. Flash chromatography refers to the procedure developed by Still and coworkers [483] and was carried out on sorbsil C60 silica gel.

The NMR spectra for the nuclei $^{31}P$, $^{1}H$ and $^{13}C$ were recorded on a Jeol FX-90Q GX270, GX400 or GLI EX400 spectrometers. Chemical shifts were measured in parts per million (ppm) relative to tetramethylsilane (TMS), deuterium oxide ($D_2O$) or dimethyl sulphoxide-$d_6$ ($d_6$-DMSO). The NMR of samples recorded in $D_2O$ were approximately pH 4-5. The $^{31}P$ NMR shifts were measured in ppm relative to external 85% phosphoric acid. Coupling constants, J were measured in hertz (Hz) and the following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, ex = exchanged with $D_2O$ and Cq = quaternary carbon. Melting points (uncorrected) were determined using a Reichert-Jung Thermo Galen Kofler block, using two glass plates. Microanalysis was carried out by the University of Bath microanalysis service. Low resolution mass spectra were recorded by the University of Bath Mass Spectrometry Service using +ve and -ve Fast Atom Bombardment (FAB) with 3-nitrobenzyl alcohol (NBA) as the matrix. High resolution accurate mass spectrometry was carried out by the SERC Mass Spectrometry Service in Swansea. Optical rotations were measured using an Optical Activity Ltd. AA-10 polarimeter using 1ml, 2.5ml or 5ml cells. All rotations were measured out at ambient temperature.

Chemicals were purchased from Aldrich, Fluka, Lancaster and Fisons chemical companies. Ether refers to diethyl ether, light petroleum refers to the boiling range of 40-60°C and evaporation refers to solvent removed under reduced pressure. The term *in vacuo*, refers to the removal of solvent on a cold finger rotary evaporator at 1mmHg or below. Ether, *N,N*-dimethylformamide (DMF), dichloromethane, and ethyl acetate were distilled from phosphorus pentoxide and stored over 4Å sieves. Triethylamine and *N,N*-diisopropylethylamine were distilled from calcium hydride. *N,N*-Diisopropylamine was distilled from sodium hydroxide pellets and pyridine was distilled from potassium hydroxide pellets. Phosphorus trichloride was purified by refluxing for 1h followed by distillation and potassium *t*-butoxide was purified by sublimation at 225°C/1mmHg. Benzyl alcohol and DMSO were purchased in anhydrous form and stored over 4Å sieves. Sodium hydride was 60% pure and used in a dispersion oil.

Ion exchange chromatography was performed on an LKB-Pharmacia Medium Pressure Ion Exchange Chromatograph using Q-Sepharose and gradients of triethylammonium bicarbonate (TEAB) as eluent. Fractions containing phosphate and phosphorothioate were assayed by a modification of the Briggs phosphate test [484] in the following way. For the quantitative test, each sample was assayed by pipetting a known volume of sample into a test tube, which was heated to dryness. Three drops of concentrated sulphuric acid were added to each tube, which was heated in an oven at 175°C for 1h. The tubes were allowed to cool, and water (250μl) was added to each sample in order to dissolve the residue. A solution of ammonium molybdate (500μl) [(12.5%$^{w}/_{v}$) in water together with concentrated sulphuric acid (8ml)] was added, followed by 250μl of hydroquinone solution [(0.5%$^{w}/_{v}$) in water (20ml) and one drop of concentrated sulphuric acid] which was then shaken, and finally 250μl of sodium sulphite solution [(20%$^{w}/_{v}$) in water (20ml)] was added. The mixture was heated to reflux temperature for 10s and a blue colour indicated the presence of inorganic phosphate. For the quantitive assays the solutions were transferred to volumetric flasks and made up to 10ml with milliQ water. The UV absorbance at 340nm was recorded using 3ml matched quartz cells. The concentration was calculated from two standard curves and drawn from UV absorbance values of known concentrations of $KH_2PO_4$ which were treated as above and measured at 340nm. 0.1M and 1M HCl was in the form of an aqueous solution.

## 6.1 The Synthesis of DL-3,6-Di-*O*-Benzoyl *myo*-Inositol-1,2,4,5-Tetrakisphosphate and DL-*myo*-Inositol-1,2,4,5-Tetrakisphosphate

### 6.1.1 DL-3,6-Di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol (36)

The title compound was prepared according to Gigg and coworkers. [344]
A mixture of *myo*-inositol (100g, 0.55mol), DMF (500ml), 2,2-dimethoxypropane (400ml, 3.25mol) and toluene-*p*-sulphonic acid (2.0g, 10.5mmol), was stirred at 100-120° C for 2h. Triethylamine (20ml), was added to the clear cooled solution and the lower boiling solvents were evaporated at 50°C. Pyridine (300ml, 3.71mol) was added to the DMF solution, followed by benzoyl chloride (400ml, 2.58mol), added dropwise, with stirring at 0°C over 30min. After a further 2h the solid was collected and washed

successively with pyridine, water, acetone and ether to give the crude product **(36)**. Yield, (77.54g, 29.8%).

m.p. 328-330°C (from DMF); (lit. [344] 328-330°C).

$\delta_H$ ($CDCl_3$; 270MHz) 1.30, 1.43, 1.51, 1.64 (12H, 4s, C*Me*$_2$), 3.73 (1H, dd, J 9.34, 10.99, H-5, Ins), 4.38 (1H, dd, J 5.13, 10.26, H-1, Ins), 4.41 (1H, dd, J 3.3, 9.71, H-4, Ins), 4.79 (1H, t, J 4.4, H-2, Ins), 5.43 (1H, dd, J 4.22, 10.63, H-3, Ins), 5.61 (1H, dd, J 6.78, 11.17 H-6, Ins), 7.26-7.62 (6H, m, Ins-O-C(O)*Ph*), 8.08-8.16 (4H, m, Ins-O-C(O)*Ph*).

### 6.1.2 DL-1,4-Di-*O*-benzoyl-*myo*-inositol (272)

DL-3,6-Di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(36)** (9.36g, 20mmol) was suspended in 80% acetic acid (200ml). The mixture was heated under reflux for 0.5h, cooled, and poured into an ice-water mixture (1000ml). The precipitated solid was filtered, washed thoroughly with ether and recrystallised from DMF-water to give the title compound **(272)**. Yield, (7.25g, 93%).

m.p. 253°C (from DMF-water).
(Found: C, 61.9; H, 5.11. $C_{20}H_{20}O_8$ requires C, 61.85; H, 5.15).

$\delta_H$ ($d_6$-DMSO; 270MHz) 3.48 (1H, dt, J 4.65, 9.3, H-5, Ins), 3.73 (1H, ddd, J 2.25, 6.9, 9.52, H-3, Ins), 3.93 (1H, dt, J 5.49, 9.53, H-6, Ins), 4.07 (1H, br t, J 2.00, H-2, Ins), 4.83 (1H, dd, J 2.00, 10.00, H-1, Ins), 4.97 (1H, d, J 6.6, $D_2O$ ex, Ins-OH), 5.25-5.33 (2H, m, $D_2O$ ex, Ins-OH), 5.35 (1H, t, J 9.9, H-4, Ins), 5.38 (1H, d, J 4.2, $D_2O$ ex, Ins-OH), 7.50-7.69 (6H, m, Ins-O-C(O)*Ph*), 8.00-8.09 (4H, m, Ins-O-C(O)*Ph*).

$\delta_C$ ($d_6$-DMSO; 68MHz) 69.83, 70.77, 70.93, 73.07, 75.70, 76.28 (6d, *C*H, *myo*-inositol ring carbons), 129.34, 130.12, 130.25, 130.64, 133.85, 134.14 (6d, Ins-O-C(O)*Ph*), 131.13 (s, Cq, Ins-O-C(O)*Ph*), 166.58 (s, Cq, Ins-O-*C*(O)Ph).

m/z (+ve ion FAB) 389 [M + H, (100%)] 306 (62%) 274 (28%) 243 (20%) 199 (40%) 105 (88%).

### 6.1.3 DL-3,6-Di-*O*-benzoyl-1,2,4,5-tetrakis(diethoxyphospho)-*myo*-inositol (275)

A mixture of DL-1,4-di-*O*-benzoyl-*myo*-inositol **(272)** (0.776g, 2mmol), dry DMF (10ml) and dry *N,N*-diisopropylethylamine (2.8ml, 16mmol), was stirred under an atmosphere of nitrogen at room temperature. The solution was cooled in an ice bath and diethoxychlorophosphine **(273)** (2.32ml, 16mmol) was added dropwise over a period of 5min and then warmed to room temperature. After stirring for 1h, 70% *t*-butylhydroperoxide, (3ml, 21.8mmol) was added to the reaction mixture at -78°C to give the crude product, $R_f$ = 0.20 (ethyl acetate). The solvents were evaporated *in vacuo* and the remaining solid was partitioned between water and dichloromethane (50ml of each). The organic layer was washed with 10% sodium metabisulphite solution, brine (20ml of each) and finally water (2x20ml). The organic layer was dried over magnesium sulphate and evaporated to give a solid. The crude product was purified over silica gel (ethyl acetate) and the solvent was evaporated to give the pure title compound **(275)**. Yield, (1.547g, 83%).

m.p. 122-123°C (from ethyl acetate-hexane).
(Found: C, 46.1; H, 6.03. $C_{36}H_{56}O_{20}P_4$ requires C, 46.35; H, 6.00).

$\delta_H$ ($CDCl_3$; 400MHz) 0.82-0.88 (9H, m, Ins-O-P(O)$OCH_2CH_3$), 1.20-1.33 (15H, m, Ins-O-P(O)$OCH_2CH_3$), 3.52-3.85 (6H, m, Ins-O-P(O)$OCH_2CH_3$), 4.01-4.21 (10H, m, Ins-O-P(O)$OCH_2CH_3$), 4.79 (2H, q, J 9.46, H-1 and H-5, Ins), 5.15 (2H, q, J 9.16, 9.46, H-4, Ins), 5.25 (1H, td, J 2.44, 9.16, H-2 or H-3, Ins), 5.29 (1H, td, J 2.13, 10.07, H-2 or H-3, Ins), 5.90 (1H, t, J 10.07, H-6, Ins), 7.43-7.59 (6H, m, Ins-O-C(O)*Ph*), 8.17-8.24 (4H, m, Ins-O-C(O)*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 15.24, 15.34, 15.79, 15.89 (4q, Ins-O-P(O)$OCH_2CH_3$), 63.66, 63.79, 64.22 (3t, Ins-O-P(O)$OCH_2CH_3$), 70.02, 73.27, 75.05, 75.37, 76.35 (5d, *C*H, *myo*-inositol ring carbons), 129.02, 129.70 (2s, Cq, Ins-O-C(O)*Ph*), 128.11, 128.21, 130.12, 130.38, 133.14, 133.37 (6d, Ins-O-C(O)*Ph*), 165.32 (s, Cq, Ins-O-*C*(O)Ph).

$\delta_P$ ($CDCl_3$; 162MHz) -1.53, -2.11, -2.18, -2.49 ($^1H$-$^{31}P$ decoupled).

m/z (+ve ion FAB) 933 [M + H, (18%)] 779 (5%) 105 (100%).

### 6.1.4 DL-*myo*-Inositol-3,6-di-*O*-benzoyl-1,2,4,5-tetrakisphosphate (276)

DL-3,6-Di-*O*-benzoyl-1,2,4,5-tetrakis(diethoxyphospho)-*myo*-inositol **(275)** (0.932g, 0.1mmol) in dry dichloromethane (5ml), was stirred at room temperature under an atmosphere of nitrogen. Bromotrimethylsilane (0.264ml, 2mmol), was added to the dry dichloromethane, and the mixture was stirred overnight. The solvents were evaporated and the residue was stirred with water (2ml), for 1h. Final purification of a third of the compound was carried out by ion exchange chromatography, on Q-Sepharose, using a buffer gradient of TEAB 200-1000mmol and flow rate 5ml/min. The fractions which gave a positive Briggs test and eluted at *ca.* 500mmol buffer, were pooled to give pure **(276)**. Yield, (27μmol, 81%).

$\delta_H$ ($D_2O$; 270MHz) 4.54-4.62 (2H, m, H-1 and H-5, Ins), 4.80 (1H, H-4, Ins, obscured by HDO peak), 5.07 (1H, d, J 10.25, H-3, Ins), 5.23 (1H, d, J 10.07, H-2, Ins), 5.61 (1H, t, J 9.89, H-6, Ins), 7.47-7.66 (6H, m, Ins-O-C(O)*Ph*), 8.09-8.16 (4H, m, Ins-O-C(O)*Ph*).

$\delta_C$ ($D_2O$; 68MHz) 71.51, 72.39, 73.88, 74.95 (4d, *C*H, *myo*-inositol ring carbons), 128.56, 128.98 (2s, Cq, Ins-O-C(O)*Ph*), 128.04, 128.79, 129.54, 129.70, 133.22 (5d, *C*H, Ins-O-C(O)*Ph*), 167.52, 167.74 (2s, Cq, Ins-O-*C*(O)Ph).

$\delta_P$ ($D_2O$; 162MHz) -0.22 (d, J 9.76, -CH-O-$PO_3^{2-}$), -0.39 (d, J 10.68, -CH-O-$PO_3^{2-}$), -0.49 (d, J 11.9, -CH-O-$PO_3^{2-}$), -0.79 (d, J, 8.83, -CH-O-$PO_3^{2-}$).

m/z (-ve ion FAB) 707 [M - H, (100%)] 460 (12%) 387 (30%) 232 (95%) 177 (20%) 159 (44%) 97 (30%).

Accurate mass spectrum requires: $(M - H)^-$ = 706.9732. Found 706.9730.

### 6.1.5 DL-*myo*-Inositol-1,2,4,5-tetrakisphosphate (152)

Crude DL-*myo*-inositol-3,6-di-*O*-benzoyl-1,2,4,5-tetrakisphosphate **(276)** (0.1mmol), was heated with 1M sodium hydroxide (3ml), at 60°C for 1h. Dowex ($H^+$ form) was added with water (30ml), until the pH was ~ 6. The Dowex was filtered off, washed with water (2x20ml), and the benzoic acid was removed by washing with dichloromethane (2x30ml). The aqueous layer was then concentrated and the residue was purified by ion exchange chromatography, using a gradient of TEAB (0-1000mmol). The pure title compound **(152)** eluted at *ca.* 550mmol TEAB. Yield, (80μmol, 80%).

$\delta_H$ ($D_2O$; 400MHz) 3.70 (1H, d, J 10.07, H-3, Ins), 3.90 (1H, t, J 9.46, H-6, Ins), 3.97-4.04 (2H, m, H-1 and H-5, Ins), 4.29 (1H, q, J 9.16, H-4, Ins), 4.80 (1H, H-2, Ins, obscured by HDO peak).

$\delta_C$ ($D_2O$; 68MHz) 69.86, 70.86, 73.91, 74.88, 76.41, 77.71 (6d, *C*H, *myo*-inositol ring carbons).

$\delta_P$ ($D_2O$; 162MHz) +1.31 (d, J 8.00, -CH-O-$PO_3^{2-}$), +1.15 (d, J 7.90, -CH-O-$PO_3^{2-}$), +1.04 (d, J 9.9, -CH-O-$PO_3^{2-}$), -0.04 (d, J 6.00, -CH-O-$PO_3^{2-}$).

m/z (-ve ion FAB) 499 [M - H, (100%)] 481 (5%) 401 (5%) 154 (10%) 97 (7%).

Accurate mass spectrum requires: $(M - H)^-$ = 498.9210. Found 498.9210.

## 6.2 Synthesis of D- and L-*myo*-Inositol-1,2,4,5-Tetrakisphosphate and D-*myo*-Inositol-1,2,4,5-Tetrakisphosphorothioate

### 6.2.1 DL-1,2:4,5-Di-*O*-isopropylidene-*myo*-inositol (37)

The title compound was prepared by the method of Gigg and coworkers. [344]
A mixture of DL-3,6-di-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(36)** (40g, 84mmol), sodium hydroxide (12g, 300mmol) and methanol (500ml), was heated under reflux for 1h. The colourless solution was cooled, neutralised with carbon dioxide, diluted with water (200ml) and evaporated to dryness. The residue was extracted with dichloromethane (6x200ml), concentrated then recrystallised from ethyl acetate to give the title compound **(37)**, $R_f$ = 0.2 (ether). Yield, (17.8g, 82%).

m.p. 171-173°C (from ethyl acetate); (lit. [344] 171-173°C).

$\delta_H$ ($CDCl_3$; 400MHz) 1.38, 1.46, 1.48, 1.54 (12H, 4s, C*Me*$_2$), 2.83 (1H, d, J 8.24, $D_2O$ ex, Ins-OH), 3.20 (1H, br s, $D_2O$ ex, Ins-OH), 3.33 (1H, t, J 10.07, H-5, Ins), 3.84 (1H, t, J 9.77, H-4, Ins), 3.90 (1H, br d, J 7.94, $D_2O$ ex, dd, J 6.41, 10.68, H-6, Ins), 4.03 (1H, br dd, J 4.58, 8.55, $D_2O$ ex, dd, J 4.27, 10.07, H-3, Ins), 4.08 (1H, dd, J 5.19, 6.1, $D_2O$ ex, dd, J 5.79, 5.5, H-1, Ins) 4.49 (1H, t, J 5.5, H-2, Ins).

$\delta_C$ ($CDCl_3$; 100MHz) 25.80, 25.85, 26.85, 26.92 (4q, C*Me*$_2$), 69.70, 74.71, 77.63, 77.92, 78.14, 81.86 (6d, *C*H, *myo*-inositol ring carbons), 110.26, 112.67 (2s, Cq, *C*Me$_2$).

### 6.2.2 DL-3,6-Di-*O*-benzyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol (106)

The title compound was prepared according to Gigg and coworkers. [344]
A mixture of DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(37)** (9.1g, 35mmol), DMF (200ml) and sodium hydride (4.8g, 200mmol), was stirred at room temperature. Benzyl bromide (11.9ml, 0.1mol), was added dropwise to the stirred solution and after 2h, TLC (ether-light petroleum, 1:1) showed a new spot $R_f = 0.60$. Methanol (50ml), was added dropwise to destroy the excess sodium hydride, water (200ml) was added to the mixture and the product was extracted with ether (2x200ml). The ether layer was washed again with water (2x200ml), dried over magnesium sulphate, filtered and evaporated to give a white crystalline solid **(106)**. Yield, (14.0g, 91%).

m.p. 152-154°C (from hexane); (lit. [344] 153-155°C).

$\delta_H$ ($CDCl_3$; 270MHz) 1.31, 1.38, 1.46, 1.49 (12H, 4s, C*Me*$_2$), 3.34 (1H, t, J 9.52, H-5, Ins), 3.67 (1H, dd, J 6.4, 10.4, H-4, Ins), 3.74 (1H, dd, J 4.2, 10.0, H-3, Ins), 3.98-4.07 (2H, m, H-1 and H-6, Ins), 4.29 (1H, t, J 4.4, H-2, Ins), 4.76-4.91 (4H, m, Ins-O-C*H*$_2$Ph), 7.23-7.40 (10H, m, Ins-O-CH$_2$*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 25.81, 26.95, 27.76 (3q, C*Me*$_2$), 71.71, 71.84 (2t, Ins-O-*C*H$_2$Ph), 74.24, 76.54, 77.03, 78.72, 79.76, 80.96 (6d, *C*H, *myo*-inositol ring carbons), 109.76, 111.99 (2s, Cq, *C*Me$_2$), 127.30, 127.69, 127.79, 128.05, 128.18, 128.25 (6d, Ins-O-CH$_2$*Ph*), 137.78, 138.07 (2s, Cq, Ins-O-CH$_2$*Ph*).

### 6.2.3 DL-1,4-Di-*O*-benzyl-2,3-*O*-isopropylidene-*myo*-inositol (277)

DL-3,6-Di-*O*-benzyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(106)** (5.28g, 12mmol), was dissolved in dichloromethane (100ml), followed by the addition of a catalytic amount of toluene-*p*-sulphonic acid (20mg, 0.1mmol) and one equivalent of ethane 1,2-diol (0.565ml, 12mmol). The mixture was stirred at room temperature until the solvent became slightly turbid. TLC (ether) showed a major product $R_f = 0.30$, a trace product $R_f = 0.06$, and a trace of starting material $R_f = 0.80$. Triethylamine (2ml) was added to the reaction mixture and the solvent was evaporated. Purification by flash chromatography (dichloromethane-ethyl acetate, 1:1), gave the title compound **(277)**. Yield, (3.84g, 80%).

m.p. 160-161°C (from ethyl acetate); (lit. [349] 161-163°C).

$\delta_H$ ($CDCl_3$; 270MHz) 1.33, 1.48 (6H, 2s, C*Me*$_2$), 2.96 (1H, br s, $D_2O$ ex, Ins-OH), 3.01 (1H, br s, $D_2O$ ex, Ins-OH), 3.35 (1H, t, J 9.34, H-5, Ins), 3.51 (1H, d, J 10.25, H-3 Ins), 3.52 (1H, t, J 9.9, H-6, Ins), 3.92 (1H, t, J 9.52, H-4, Ins), 4.06 (1H, dd, J 5.31, 6.78, H-1, Ins), 4.27 (1H, dd, J 4.21, 5.13, H-2, Ins), 4.68, 4.91 (2H, AB, J 11.54, Ins-O-C*H*$_2$Ph), 4.77 (2H, s, Ins-O-C*H*$_2$Ph), 7.24-7.41 (10H, m, Ins-O-CH$_2$*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 25.88, 27.99 (2q, C*Me*$_2$), 72.55, 73.27 (2t, Ins-O-*C*H$_2$Ph), 71.51, 72.97, 73.98, 76.93, 79.17, 81.89 (6d, *C*H, *myo*-inositol ring carbons), 109.85 (s, Cq, *C*Me$_2$), 127.66, 127.96, 128.02, 128.31, 128.44 (5d, *C*H, Ins-O-CH$_2$*Ph*), 137.78, 138.07 (2s, Cq, Ins-O-CH$_2$*Ph*).

### 6.2.4 DL-3,6-Di-*O*-benzyl-1,2-*O*-isopropylidene-4,5-di-*O*-*p*-methoxybenzyl-*myo*-inositol (278)

A mixture of DL-1,4-di-*O*-benzyl-2,3-*O*-isopropylidene-*myo*-inositol **(277)** (2.8g, 7mmol) and sodium hydride (0.72g, 30mmol) was dissolved in dry DMF (50ml). *p*-Methoxybenzyl chloride (2.9ml, 20mmol), was added dropwise at room temperature and stirred for 2h. TLC (ether-petroleum ether, 2:1) showed a new spot, $R_f = 0.40$. The excess sodium hydride was destroyed with methanol (10ml) and the solvents were evaporated *in vacuo*. The remaining syrup was partitioned between water (100ml) and ether (100ml), washed with 0.1M HCl (100ml), a saturated aqueous solution of sodium hydrogen carbonate (100ml), and water (100ml). The organic layer was dried over magnesium sulphate, the remaining syrup was purified by flash chromatography (ether-petroleum ether, 2:1) and the product was isolated as a syrup. Yield, (3.60g, 80%).

(Found: C, 73.0; H, 6.64. $C_{42}H_{48}O_8$ requires C, 73.12; H, 6.87).

$\delta_H$ ($CDCl_3$; 270MHz) 1.35, 1.51 (6H, 2s, C*Me*$_2$), 3.39 (1H, t, J 8.79, H-5, Ins), 3.67 (1H, dd, J 3.61, 8.8, H-3 or H-1, Ins), 3.74-3.80 (1H, obscured, H-3 or H-1, Ins), 3.77 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.79 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.92 (1H, t, J 8.61, H-4 or H-6, Ins), 4.09 (1H, t, J 6.60, H-4 or H-6, Ins), 4.25 (1H, dd, J 4.03, 5.31, H-2, Ins), 4.71-4.88 (8H, m, Ins-O-C$H_2$Ph), 6.84 (4H, 2d, J 9.1 Ins-O-$CH_2$*Ph*OMe), 7.21-7.41 (14H, m, Ins-O-$CH_2$*Ph* and Ins-O-$CH_2$*Ph*OMe).

$\delta_C$ ($CDCl_3$; 68MHz) 25.53, 27.59 (2q, C*Me*$_2$), 55.04 (q, Ins-O-$CH_2$PhO*Me*), 73.10, 73.65, 74.73 (3t, Ins-O-*C*H$_2$Ph and Ins-O-*C*H$_2$PhOMe), 74.37, 77.00, 78.91, 80.47, 81.70, 82.35 (6d, *C*H, *myo*-inositol ring carbons), 109.56 (s, Cq, *C*Me$_2$), 113.52, 113.58, 114.10, 127.30, 127.63, 127.72, 127.82, 128.05, 128.21, 129.44 (10d, Ins-O-$CH_2$*Ph* and Ins-O-$CH_2$*Ph*OMe), 131.78, 130.61 (2s, Cq, Ins-O-$CH_2$*Ph*OMe), 138.04, 138.40 (2s, Cq, Ins-O-$CH_2$*Ph*), 158.96 (s, Cq, Ins-O-$CH_2$*Ph*OMe).

m/z (-ve ion FAB) 549 [M - benzyl (8%)] 519 [M - *p*-methoxybenzyl (40%)] 335 (10%) 258 (30%) 137 [-O-$CH_2$PhOMe (100%)] 107 [-O-$CH_2$Ph (70%)].

### 6.2.5 DL-1,4-Di-*O*-benzyl-5,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol (279)

DL-3,6-Di-*O*-benzyl-1,2-*O*-isopropylidene-4,5-di-*O*-*p*-methoxybenzyl-*myo*-inositol **(278)** (2.25g, 3.5mmol), was dissolved in a mixture of methanol-1M HCl (9:1, 30ml), which was kept at 50°C for 30min. TLC (ether) showed a new spot $R_f$ = 0.40. Sodium hydrogen carbonate (2g), was added and the solvents were evaporated under reduced pressure. The product was extracted with dichloromethane (3x100ml), and the organic solvent was evaporated to give a solid. The crude product was purified by flash chromatography (ether-chloroform, 3:1), to give the title compound **(279)**. Yield, (1.9g, 90%).

m.p. 130-132°C (from ethyl acetate-hexane); (lit. [474] 130.4-130.6°C).

$\delta_H$ ($CDCl_3$; 270MHz) 2.53 (1H, d, J 4.4, $D_2O$ ex, Ins-OH), 2.62 (1H, s, $D_2O$ ex, Ins-OH), 3.43 (2H, overlapping, H-3 and H-1, Ins), 3.44 (1H, t, J 9.34, H-5, Ins), 3.78 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.79 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.81 (1H, t, J 9.34, H-4 or H-6, Ins), 3.94 (1H, t, J 9.53, H-4 or H-6, Ins), 4.25 (1H, br s, H-2, Ins), 4.70-4.96 (8H, m, Ins-O-C$H_2$Ph), 6.83 (2H, d, J 8.8, Ins-O-$CH_2$*Ph*OMe), 6.84 (2H, d, J 8.8, Ins-O-$CH_2$*Ph*OMe), 7.21-7.36 (14H, m, Ins-O-$CH_2$*Ph* and Ins-O-$CH_2$*Ph*OMe).

$\delta_C$ ($CDCl_3$; 68MHz) 55.04 (q, Ins-O-$CH_2$PhO*Me*), 72.68, 75.34, 75.50 (3t, Ins-O-*C*$H_2$Ph and Ins-O-*C*$H_2$PhOMe), 69.15, 71.71, 80.01, 81.28, 81.37, 82.93 (6d, *C*H, *myo*-inositol ring carbons), 113.74, 127.82, 127.89, 128.54, 129.41, 129.54 (5d, Ins-O-$CH_2$*Ph* and Ins-O-$CH_2$*Ph*OMe), 130.68, 130.84 (2s, Cq, Ins-O-$CH_2$*Ph*OMe), 138.49, 138.81 (2s, Cq, Ins-O-$CH_2$*Ph*), 159.12 (s, Cq, Ins-O-$CH_2$*Ph*OMe).

m/z (-ve ion FAB) 753 [M + NBA, (40%)] 599 [M - H, (100%)] 509 [M - benzyl, (10%)] 479 [M - *p*-methoxybenzyl, (20%)] 335 (15%) 137 [-O-$CH_2$PhOMe, (30%)] 107 [-O-$CH_2$Ph, (30%)].

### 6.2.6 D-(281) And L-1-*O*-[*S*-(+)-*O*-acetylmandelyl]-3,6-di-*O*-benzyl-4,5-di-*O*-*p*-methoxybenzyl-*myo*-inositol (282)

A mixture of DL-1,4-di-*O*-benzyl-5,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol **(279)** (2.5g, 4.17mmol), *S*-(+)-*O*-acetylmandelic acid **(280)** (0.835g, 4.3mmol) and DMAP (0.03g, 0.25mmol), was stirred in dichloromethane, (15ml) at -20°C (dry ice alone). DCC (0.877g, 4.33mmol) in dichloromethane (5ml) was added dropwise over 90min with stirring. The reaction mixture was stirred at room temperature overnight after which, TLC (chloroform-acetone, 30:1) showed two products, $R_f$ = 0.44 and 0.34. The mixture was filtered through celite and washed thoroughly with dichloromethane (100ml). The solvent was evaporated to give a white solid and the individual diastereoisomers were separated by flash chromatography, (chloroform-acetone, 30:1) to give **(281)** $R_f$ = 0.44, (36% yield) and **(282)** $R_f$ = 0.34, (37% yield).

**(281)** m.p. 120-121°C (from ethanol).
**(282)** m.p. 147-148°C (from ethanol).
**(281)** $[\alpha]_D$ = +12° (c = 1 in $CH_2Cl_2$).
**(282)** $[\alpha]_D$ = +42° (c = 1 in $CH_2Cl_2$).
**(281)** (Found: C, 71.1; H, 6.27. $C_{46}H_{48}O_{11}$ requires C, 71.10; H, 6.23).
**(282)** (Found: C, 70.8; H, 6.22. $C_{46}H_{48}O_{11}$ requires C, 71.10; H, 6.23).

**(281)** $\delta_H$ ($CDCl_3$; 400MHz) 2.16 (1H, s, $D_2O$ ex, Ins-OH), 2.19 (3H, s, Ins-$O_2$C-CH-(O*Ac*)Ph), 3.44 (1H, dd, J 2.45, 9.46, H-3, Ins), 3.49 (1H, t, J 9.46, H-5, Ins), 3.77 (3H, s, Ins-O$CH_2$PhO*Me*), 3.78 (3H, s, Ins-O$CH_2$PhO*Me*), 3.91 (1H, t, J 9.46, H-4, Ins), 4.05 (1H, t, J 10.07, H-6, Ins), 4.15 (1H, br d, J 1.83, H-2, Ins), 4.61-4.81 (9H, m, Ins-

$OCH_2Ph$, Ins-$OCH_2$PhOMe, and H-1, Ins), 5.94 (1H, s, Ins-$O_2C$-C*H*-(OAc)Ph), 6.82 (2H, d, J 8.54, Ins-$OCH_2$*Ph*OMe), 6.83 (2H, d, J 8.85, Ins-$OCH_2$*Ph*OMe), 7.17-7.44 (19H, m, Ins-$OCH_2$*Ph*, Ins-$OCH_2$*Ph*OMe and Ins-$O_2C$-CH-(OAc)*Ph*).

$\delta_C$ ($CDCl_3$; 100MHz) 20.70 (q, Ins-$O_2C$-CH-(O*Ac*)Ph, -OC(O)$CH_3$), 55.25 (q, Ins-$OCH_2$PhO*Me*), 67.32, 74.78, 75.25, 78.42, 79.61, 80.72, 82.73 (7d, *C*H, *myo*-inositol ring carbons and Ins-$O_2C$-*C*H-(OAc)Ph), 72.80, 75.46, 75.58 (3t, Ins-O$CH_2$Ph and Ins-O$CH_2$PhOMe), 113.77 (d, Ins-$OCH_2$*Ph*OMe), 127.34, 127.52, 127.76, 127.91, 128.29, 128.47, 128.82, 129.26, 129.42, 129.55 (10d, Ins-$OCH_2$*Ph*, Ins-$OCH_2$*Ph*OMe and Ins-$O_2C$-CH-(OAc)*Ph*), 130.76, 130.81, 133.37, 137.71, 138.31 (5s, Cq, Ins-$OCH_2$*Ph*, Ins-$OCH_2$*Ph*OMe and Ins-$O_2C$-CH-(OAc)*Ph*), 159.14, 159.18 (2s, Ins-$OCH_2$*Ph*OMe), 168.27, 170.75 (2s, Cq, Ins-$O_2$*C*-CH-(O*Ac*)Ph, -O*C*(O)$CH_3$).

m/z (-ve ion FAB) 929 [M + NBA, (30%)] 775 [M - H, (58%)] 599 (50%) 193 (55%) 149 (100%).

**(282)** $\delta_H$ ($CDCl_3$; 400MHz) 2.17 (3H, s, Ins-$O_2C$-CH-(O*Ac*)Ph), 2.69 (1H, s, $D_2O$ ex, Ins-OH), 3.43 (1H, t, J 9.46, H-5, Ins), 3.49 (1H,dd, J 2.75, 9.77, H-3, Ins), 3.75 (3H, s, Ins-$OCH_2$PhO*Me*), 3.77 (3H, s, Ins-$OCH_2$PhO*Me*), 3.93 (1H, t, J 9.77, H-4, Ins), 4.00 (1H, t, J 9.77, H-6, Ins), 4.14, 4.46 (2H, AB, J 10.99, Ins-O$CH_2$Ph or Ins-O$CH_2$PhOMe), 4.40 (1H, br d, J 1.83, H-2, Ins), 4.61-4.84 (7H, m, Ins-O$CH_2$Ph, Ins-O$CH_2$PhOMe, and H-1, Ins), 5.98 (1H, s, Ins-$O_2C$-C*H*-(OAc)Ph), 6.75 (2H, d, J 8.54, Ins-$OCH_2$*Ph*OMe), 6.82 (2H, d, J 8.85, Ins-$OCH_2$*Ph*OMe), 6.83-7.46 (19H, m, Ins-$OCH_2$*Ph*, Ins-$OCH_2$*Ph*OMe and Ins-$O_2C$-CH-(OAc)*Ph*).

$\delta_C$ ($CDCl_3$; 100MHz) 20.65 (q, Ins-$O_2C$-CH-(O*Ac*)Ph, -OC(O)$CH_3$), 55.23, 55.27 (2q, Ins-$OCH_2$PhO*Me*), 67.43, 74.94, 75.40, 78.40, 79.79, 80.74, 82.70 (7d, *C*H, *myo*-inositol ring carbons and Ins-$O_2C$-*C*H-(OAc)Ph), 72.80, 74.98, 75.58 (3t, Ins-O$CH_2$Ph and Ins-O$CH_2$PhOMe), 113.68, 113.75 (2d, Ins-$OCH_2$*Ph*OMe), 127.19, 127.25, 127.89, 128.02, 128.49, 128.84, 129.41, 129.50 (8d, Ins-$OCH_2$*Ph*, Ins-$OCH_2$*Ph*OMe and Ins-$O_2C$-CH-(OAc)*Ph*), 130.65, 130.83, 132.92, 137.63, 138.20 (5s, Cq, Ins-$OCH_2$*Ph*, Ins-$OCH_2$*Ph*OMe and Ins-$O_2C$-CH-(OAc)*Ph*), 159.08, 159.16 (2s, Ins-$OCH_2$*Ph*OMe), 168.58, 170.66 (2s, Cq, Ins-$O_2$*C*-CH-(O*Ac*)Ph, -O*C*(O)$CH_3$).

m/z (-ve ion FAB) 929 [M + NBA, (15%)] 775 [M - H, (28%)] 599 (25%) 193 (55%) 149 (100%).

### 6.2.7 D-3,6-Di-*O*-benzyl-4,5-di-*O*-*p*-methoxybenzyl-*myo*-inositol (283)

A mixture of D-1-*O*-[*S*-(+)-*O*-acetylmandelyl]-3,6-di-*O*-benzyl-4,5-di-*O*-*p*-methoxy benzyl-*myo*-inositol **(281)** (0.956g, 1.23mmol), sodium hydroxide (0.40g, 10mmol) and methanol (100ml) was heated at reflux temperature for 30min. The mixture was cooled and neutralised with carbon dioxide. The resulting solid was diluted with water (50ml) and evaporated to dryness *in vacuo*. The crude product was extracted with dichloromethane (4x100ml) which was then evaporated to give a solid **(283)**, $R_f = 0.40$ (ether). Yield, (0.729g, 99%).

m.p. 133-134°C (from ethyl acetate-hexane).
$[\alpha]_D = -25°$ (c = 1 in $CH_2Cl_2$).
(Found: C, 72.1; H, 6.77. $C_{36}H_{40}O_8$ requires C, 71.98; H, 6.71).

The mass spectrum and NMR data were identical to **(279)**.

### 6.2.8 L-3,6-Di-*O*-benzyl-4,5-di-*O*-*p*-methoxybenzyl-*myo*-inositol (284)

A mixture of L-1-*O*-[*S*-(+)-*O*-acetylmandelyl]-3,6-di-*O*-benzyl-4,5-di-*O*-*p*-methoxy benzyl-*myo*-inositol **(282)** (0.929g, 1.19mmol), sodium hydroxide (0.40g, 10mmol) and methanol (100ml) was heated at reflux temperature for 30min. Work up as for the D-enantiomer gave the title compound **(284)** $R_f = 0.40$ (ether). Yield, (0.655g, 91%).

m.p. 133-134°C (from ethyl acetate-hexane).
$[\alpha]_D = +25°$ (c = 1 in $CH_2Cl_2$)
(Found: C, 72.0; H, 6.86. $C_{36}H_{40}O_8$ requires C, 71.98; H, 6.71).

The mass spectrum and NMR data were identical to **(279)**.

### 6.2.9 D-3,6-Di-*O*-benzyl-*myo*-inositol (285)

D-3,6-Di-*O*-benzyl-4,5-di-*O*-*p*-methoxybenzyl-*myo*-inositol **(283)**, (0.624g, 1.04mmol) was suspended in 1M HCl-ethanol (60ml, 1:2). The mixture was heated at reflux temperature for 4h, cooled and the solvents were evaporated *in vacuo*. The resulting solid was filtered and washed with water (10ml) and ether (2x10ml). The solid was then recrystallised from ethanol to give the pure title compound **(285)**, $R_f$ = 0.60 (chloroform-methanol, 6:1). Yield, (0.323g, 86%).

m.p. 172-173°C (from ethanol).
$[\alpha]_D$ = +16° (c = 1 in methanol).
(Found: C, 66.6; H, 6.73. $C_{20}H_{24}O_6$ requires C, 66.65; H, 6.71).

$\delta_H$ ($d_6$-DMSO; 400MHz) 3.11 (1H, dd, J 2.44, 9.77, H-3, Ins), 3.15 (1H, dt, J 4.89, 8.85, $D_2O$ ex, t, J 9.15, H-5, Ins), 3.31 (1H, ddd, J 2.44, 6.72, 9.46, $D_2O$ ex, dd, J 2.44, 9.77, H-1, Ins), 3.44 (1H, t, J 9.46, H-6, Ins), 3.60 (1H, dt, J 2.44, 5.8, $D_2O$ ex, t, J 2.44, H-2, Ins), 4.51, 4.60 (2H, AB, J 12.21, Ins-O-C$H_2$Ph), 4.67 (1H, d, J 6.71, $D_2O$ ex, Ins-OH), 4.74-4.81 (4H, m, Ins-OH and Ins-O-C$H_2$Ph), 4.84 (1H, d, J 4.89, $D_2O$ ex, Ins-OH), 7.11-7.44 (10H, m, Ins-O-$CH_2$*Ph*).

$\delta_C$ ($d_6$-DMSO; 100MHz) 69.73, 72.25, 73.59, 75.03, 79.79, 81.82 (6d, *C*H, *myo*-inositol ring carbons), 70.72, 71.43 (2t, Ins-O-*C*$H_2$Ph), 126.92, 127.08, 127.48, 127.52, 127.63, 127.85, 127.99 (7d, Ins-O-$CH_2$*Ph*), 139.32, 139.94 (2s, Cq, (7d, Ins-O-$CH_2$*Ph*).

m/z (-ve ion FAB) 513 [M + NBA, (100%)] 359 [M - H, (75%)] 291 (50%) 228 (30%).

### 6.2.10 L-3,6-Di-*O*-benzyl-*myo*-inositol (286)

L-3,6-Di-*O*-benzyl-4,5-di-*O*-*p*-methoxybenzyl-*myo*-inositol **(284)**, (0.590g, 0.98mmol) was suspended in 1M HCl-ethanol (60ml, 1:2). The mixture was heated at reflux temperature for 4h, cooled and the solvents were evaporated *in vacuo*. The resulting solid was filtered and washed with water (10ml) and ether (2x10ml). The solid was then recrystallised from ethanol to give the pure title compound **(286)**, $R_f$ = 0.60 (chloroform-methanol, 6:1). Yield, (0.293g, 83%).

m.p. 172-173°C (from ethanol).
$[\alpha]_D$ = -16° (c = 1 in methanol).
(Found: C, 66.4; H, 6.73. $C_{20}H_{24}O_6$ requires C, 66.65; H, 6.69).

The mass spectrum and NMR data were identical to **(285)**.

### 6.2.11 *N,N*-Diisopropylaminodichlorophosphine (289)

The title compound was prepared according to Tanaka and coworkers. [476]
Dry *N,N*-diisopropylamine **(288)** (141ml, 1.0mol) in dry ether (150ml) was added dropwise to a stirred solution of phosphorus trichloride **(287)** (44ml, 0.5mol) in dry ether (100ml) at -78°C. More dry ether (500ml) was added and the mixture was stirred for a further 15h at room temperature. The precipitated amine salt was then filtered and washed with 500ml of ether and evaporated to give an oil. The crude mixture was distilled, b.p. 62-63°C at 7mmHg (lit. b.p. 62-63°C at 7mmHg). Yield, (68.3g, 68%). The product **(289)** was stored at -20°C.

$\delta_H$ ($CDCl_3$; 90MHz) 1.27 (12H, d, J 6.78, $(Me)_2C(H)N\text{-}PCl_2$), 3.93 (2H, septet, J 7.44, $(Me)_2C(H)N\text{-}PCl_2$).

$\delta_P$ ($CDCl_3$; 36.2MHz) +169.4 ($^1H$-decoupled).

### 6.2.12 Bis(benzyloxy)diisopropylaminophosphine (96)

The title compound was prepared by the method of Yu and Fraser-Reid. [375]
A mixture of *N,N*-diisopropylaminodichlorophosphine **(289)** (23.59g, 0.116mol) in dry dichloromethane and dry triethylamine (34.8ml, 0.249mol) was stirred at -78°C under an atmosphere of nitrogen. Anhydrous benzyl alcohol (23.8ml, 0.23mol) was added dropwise and stirred for a further 2h. The amine salt was filtered and washed with a further two portions of dichloromethane (2x200ml). The organic layer was then washed with saturated sodium hydrogen carbonate (300ml) and water (300ml). The organic layer was dried over magnesium sulphate, filtered and evaporated to give a thick oil $R_f = 0.78$ (hexane-triethylamine, 10:1) and the title compound **(96)** was pure by NMR. Yield, (38.46g, 96%).

$\delta_H$ ($CDCl_3$; 270MHz) 1.20 (12H, d, J 6.78, $(Me)_2C(H)N\text{-}PCl_2$), 3.60-3.80 (2H, m, $(Me)_2C(H)N\text{-}POCH_2Ph$), 4.65-4.82 (4H, m, $\text{-}CH_2Ph$), 7.22-7.36 (10H, m, $\text{-}CH_2Ph$).

$\delta_C$ ($CDCl_3$; 68MHz) 24.48, 24.61 (2q, $(Me)_2C(H)N\text{-}POCH_2Ph$), 42.9 (d, $(Me)_2C(H)N\text{-}POCH_2Ph$), 65.16, 65.45 (2t, $\text{-}CH_2Ph$), 126.88, 127.11, 128.11 (3d, $\text{-}CH_2Ph$).

$\delta_P$ ($CDCl_3$; 36.2MHz) +147.86 ($^{31}P\text{-}^1H$-decoupled).

### 6.2.13 D-3,6-Di-*O*-benzyl-1,2,4,5-tetrakis[di(benzyloxyphospho)]-*myo*-inositol (293)

A mixture of bis(benzyloxy)diisopropylaminophosphine **(96)** (0.69g, 2mmol) and 1*H*-tetrazole (0.28g, 4mmol) in dry dichloromethane (5ml), was stirred at room temperature for 15min in order to form the tetrazolide intermediate **(290)**. D-3,6-Di-O-benzyl-*myo*-inositol **(285)** (0.108g, 0.30mmol), was added to **(290)**, and stirred for a further 10min. The reaction mixture was cooled to 0°C and *m*CPBA (0.8g, 2.3mmol), (50-60%) was added and stirred for a further 30min. The mixture was diluted with ethyl acetate (50ml) and washed with 10% sodium metabisulphite (50ml), 1M HCl and a saturated solution of sodium hydrogen carbonate then brine and water (50ml of each). The organic layer was separated then dried over magnesium sulphate and evaporated to give a syrup. The product was purified by flash chromatography, $R_f$ = 0.20, (chloroform-acetone, 5:1) then (ethyl acetate-pentane, 2:1), in order to obtain the pure title compound **(293)** as a syrup. Yield, (0.395g, 94%).

$[\alpha]_D$ = -3.5° (c = 2 in $CH_2Cl_2$).
(Found: C, 65.2; H, 5.54. $C_{76}H_{76}O_{18}P_4$ requires C, 65.14; H, 5.47).

$\delta_H$ ($CDCl_3$; 400MHz) 3.55 (1H, d, J 9.76, H-3, Ins), 3.98 (1H, t, J 9.46, H-6, Ins), 4.41 (1H, t, J 9.46, H-5, Ins), 4.48-5.11 (22H, m, Ins-O-(O)PO-C*H*$_2$Ph, Ins-O-C*H*$_2$Ph, H-1, H-4, Ins), 5.43 (1H, d, J 8.86, H-2, Ins), 6.94-7.41 (50H, m, Ins-O-(O)PO-CH$_2$*Ph*, Ins-O-CH$_2$*Ph*).

$\delta_C$ ($CDCl_3$; 100MHz) 69.18, 69.23, 69.29, 69.24, 69.42, 69.47, 69.53, 69.60, 69.65, 74.37 (10t, Ins-O-(O)PO-*C*H$_2$Ph, Ins-O-*C*H$_2$Ph), 72.24, 74.37, 74.63, 75.43, 77.38, 78.66 (6d, *C*H *myo*-inositol ring carbons), 127.19, 127.39, 127.57, 127.74, 127.85, 127.96, 128.09, 128.16, 128.29, 128.45, 128.51, 128.65 (12d, Ins-O-(O)PO-CH$_2$*Ph*, Ins-O-CH$_2$*Ph*), 135.48, 135.55, 135.60, 135.73, 135.78, 135.84, 135.91, 136.01, 136.46, 137.94 (10s, Cq, Ins-O-(O)PO-CH$_2$*Ph*, Ins-O-CH$_2$*Ph*).

$\delta_P$ ($CDCl_3$; 162MHz) -1.16, -1.66, -1.71, -2.07 ($^{31}P$-$^{1}H$-decoupled).

m/z (+ve ion FAB) 1401 [M + H, (7%)] 181 (5%) 107 (2%) 91 (100%).

### 6.2.14 L-3,6-Di-*O*-benzyl-1,2,4,5-tetrakis[di(benzyloxyphospho)]-*myo*-inositol (294)

A mixture of bis(benzyloxy)diisopropylaminophosphine **(96)** (0.69g, 2mmol) and 1*H*-tetrazole (0.28g, 4mmol) in dry dichloromethane (5ml), was stirred at room temperature for 15min in order to form the tetrazolide intermediate **(290)**. L-3,6-Di-O-benzyl-*myo*-inositol **(286)** (0.108g, 0.30mmol), was added to **(290)**, and stirred for a further 10min. The reaction was cooled to 0°C and *m*CPBA (0.8g, 2.3mmol), (50-60%) was added and stirred for a further 30min. The product **(294)** was extracted and purified by chromatography in the same way as **(293)**. Yield, (0.37g, 88%).

$[\alpha]_D$ = +3.3° (c = 1.26 in $CH_2Cl_2$).
(Found: C, 65.0; H, 5.72. $C_{76}H_{76}O_{18}P_4$ requires C, 65.14; H, 5.47).

The mass spectrum and NMR data were identical to **(293)**.

### 6.2.15 D-*myo*-Inositol 1,2,4,5-tetrakisphosphate (295)

Ammonia (80ml), was distilled into a three neck flask and small pieces of freshly cut sodium metal (0.80g, 34.8mmol), were added until the solution remained blue. The dry-ice condenser was moved across to the reaction flask and ammonia (40ml), was gently transferred to the flask by heating. Small slithers of sodium (0.40g, 17.4mmol), were added to the ammonia until the colour remained blue once again. 3,6-Di-*O*-benzyl-1,2,4,5-tetrakis[di(benzyloxyphospho)]-*myo*-inositol **(293)** (0.178g, 126μmol), was dissolved in dry dioxan (1ml), which was then added to the sodium in liquid ammonia. The reaction was left for 2min and quenched with methanol (20ml). The ammonia was evaporated under a stream of nitrogen and MilliQ water was then added to the residue which was evaporated to dryness *in vacuo*. The deprotected phosphate **(295)** was purified by ion exchange chromatography on Q-Sepharose using a gradient of TEAB buffer 0-1000mmol and eluted at *ca.* 800mmol. Yield, (50.02μmol, 40%).

$[\alpha]_D$ = -27.2° (c = 0.50 in TEAB, pH = 8.6).

$\delta_H$ ($D_2O$; 400MHz) 3.59 (1H, d, J 9.77, H-3, Ins), 3.76 (1H, t, J 9.46, H-6, Ins), 3.90 (2H, q, J 9.15, H-1, H-5, Ins), 4.16 (1H, q, J 9.46, H-4, Ins), 4.59 (1H, d, J 9.77, H-2, Ins).

$\delta_P$ ($D_2O$; 162MHz) +0.65 (d, J 9.28, -CH-O-$PO_3^{2-}$), +0.27 (d, J 9.04, -CH-O-$PO_3^{2-}$), -0.01 (d, J 9.04, -CH-O-$PO_3^{2-}$), -0.33 (d, J, 8.06, -CH-O-$PO_3^{2-}$).

m/z (-ve ion FAB) 499 [M - H, (100%)] 419 (5%) 159 (10%) 97 (9%).

Accurate mass spectrum requires: $(M - H)^-$ = 498.9208. Found 498.9226.

### 6.2.16 L-*myo*-Inositol 1,2,4,5-tetrakisphosphate (296)

L-3,6-Di-*O*-benzyl-1,2,4,5-tetrakis[di(benzyloxyphospho)]-*myo*-inositol **(294)** (0.10g, 71μ mol), was deprotected as for **(295)** to give pure L-*myo*-inositol 1,2,4,5-tetrakisphosphate **(296)** after ion exchange chromatography. Yield, (15.56μmol, 22%). The NMR spectra were slightly different due to the different pH of product in the NMR tube.

$[\alpha]_D$ = +25.8° (c = 0.31 in TEAB, pH = 8.6).

$\delta_H$ ($D_2O$; 270MHz) 3.72 (1H, d, J 9.71, H-3, Ins), 3.90 (1H, t, J 9.53, H-6, Ins), 4.03 (2H, q, J 9.34, H-1, H-5, Ins), 4.30 (1H, q, J 9.52, H-4, Ins), 4.71 (1H, bd, J 9.71, H-2, Ins).

$\delta_P$ ($D_2O$; 109MHz) +1.78 (d, J 10.1, -CH-O-$PO_3^{2-}$), +1.44 (d, J 6.7, -CH-O-$PO_3^{2-}$), +1.20 (d, J 6.7, -CH-O-$PO_3^{2-}$), +0.67 (d, J, 6.7, -CH-O-$PO_3^{2-}$).

m/z (-ve ion FAB) 499 [M - H, (100%)] 419 (10%) 159 (10%) 97 (10%).

Accurate mass spectrum requires: $(M - H)^-$ = 498.9208. Found 498.9187.

### 6.2.17 D-3,6-Di-*O*-benzyl-1,2,4,5-tetrakis[di(benzyloxyphosphorothio)]-*myo*-inositol (297)

A mixture of bis(benzyloxy)diisopropylaminophosphine **(96)** (0.69g, 2mmol) and 1*H*-tetrazole (0.28g, 4mmol) in dry dichloromethane (5ml), was stirred at room temperature for 15min in order to form the tetrazolide intermediate **(290)**. D-3,6-Di-O-benzyl-*myo*-inositol **(285)** (0.108g, 0.30mmol), was added to **(290)**, and stirred for a further 10min. The dichloromethane was evaporated to give a thick syrup, pyridine (1ml), dry DMF (2ml) and sulphur (0.096g, 3mmol) were added to the syrup and the mixture was then stirred for 5min after which time sulphoxidation was complete by NMR and TLC, $R_f$ = 0.50 (ether-pentane, 2:1). The excess sulphur was then filtered off, the solvents were evaporated *in vacuo*, and the syrup was dissolved in ethyl acetate (20ml). The solution was washed with 0.1M HCl, TEAB, brine and finally water (20ml each). The organic layer was dried over magnesium sulphate, filtered and purified by flash chromatography (ether-pentane, 2:1), to give **(297)** as a syrup. Yield, (0.373g, 85%).

$[\alpha]_D$ = -5.3° (c = 1.9 in $CH_2Cl_2$).
(Found: C, 62.1; H, 5.44. $C_{76}H_{76}O_{18}P_4S_4$ requires C, 62.29; H, 5.23).

$\delta_H$ ($CDCl_3$; 400MHz) 3.61 (1H, d, J 9.77, H-3, Ins), 4.07 (1H, t, J 9.16, H-6, Ins), 4.39 (1H, t, J 11.29, H-5, Ins), 4.50-5.17 (21H, m, Ins-O-(S)PO-C$H_2$Ph, Ins-O-C$H_2$Ph, H-1, H-4, Ins), 5.28 (1H, q, J 9.15, H-4, Ins), 5.58 (1H, d, J 8.86, H-2, Ins), 6.94-7.41 (50H, m, Ins-O-(S)PO-$CH_2$*Ph*, Ins-O-$CH_2$*Ph*).

$\delta_C$ ($CDCl_3$; 100MHz) 69.58, 69.66, 69.73, 70.08, 71.94, 72.01, 73.95, 74.01 (8t, Ins-O-(S)PO-*C*$H_2$Ph, Ins-O-*C*$H_2$Ph), 75.27, 75.35, 75.44, 77.80, 78.44, 79.42 (6d, *C*H *myo*-inositol ring carbons), 126.37, 126.86, 127.56, 127.72, 127.91, 127.99, 128.22, 128.34 (8d, Ins-O-(S)PO-$CH_2$*Ph*, Ins-O-$CH_2$*Ph*), 135.53, 135.59, 135.71, 135.77, 135.88, 135.97, 136.52, 137.36 (8s, Cq, Ins-O-(S)PO-$CH_2$*Ph*, Ins-O-$CH_2$*Ph*).

$\delta_P$ ($CDCl_3$; 162MHz) +67.70, +68.31, +68.51, +68.96 ($^{31}$P-$^1$H-decoupled).

m/z (-ve ion FAB) 1371 [M - Bn, (7%)] 1281 [M - 2Bn, (1.5%)] 695 (0.5%) 479 (10%) 293 (100%) 96 (5%).

### 6.2.18 D-*myo*-Inositol 1,2,4,5-tetrakisphosphorothioate (298)

D-3,6-Di-*O*-benzyl-1,2,4,5-tetrakis[di(benzyloxyphosphorothio)]-*myo*-inositol **(297)** (0.090g, 61.4μmol), was deprotected as for **(295)** to give pure D-*myo*-inositol 1,2,4,5-tetrakisphosphorothioate **(298)** after ion exchange chromatography on Q-Sepharose Fast Flow, using a TEAB buffer gradient of 0-1000mmol. Yield, (20.4μmol, 33.2%).

$[\alpha]_D$ = -25.8° (c = 0.31 in TEAB, pH = 8.6).

$\delta_H$ ($D_2O$; 400MHz) 3.61 (1H, d, J 9.46, H-3, Ins), 3.86 (1H, t, J 9.46, H-6, Ins), 4.18 (2H, q, J 9.77, H-1, H-5, Ins), 4.46 (1H, q, J 11.29, H-4, Ins), 4.83 (1H, d, J 11.59, H-2, Ins).

$\delta_P$ ($D_2O$; 162MHz) +49.14 (d, J 11.54, -CH-O-$P(S)O_2^{2-}$), +50.20 (d, J 11.54, -CH-O-$P(S)O_2^{2-}$), +50.64 (d, J 10.26, -CH-O-$P(S)O_2^{2-}$), +51.47 (d, J, 12.18, -CH-O-$P(S)O_2^{2-}$).

m/z (-ve ion FAB) 499 [M - H, (100%)] 419 (10%) 159 (10%) 97 (10%).

Accurate mass spectrum requires: $(M - H)^-$ = 498.9208. Found 498.9187.

## 6.3 Establishing the Absolute Configuration of D-3,6-Di-*O*-Benzyl-*myo*-Inositol

### 6.3.1 Synthesis of D-3,6-di-*O*-[*S*-(+)-*O*-acetylmandelyl]-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol (299)

A mixture of DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(37)** (2.08g, 8mmol), DCC (4.13g, 20mmol) and DMAP (0.05g, 0.4mmol) in dry dichloromethane (50ml) was stirred at 0°C under an atmosphere of nitrogen. *S*-(+)-*O*-Acetylmandelic acid (3.88g, 20mmol) in dry dichloromethane (30ml) was added dropwise over 15min and the mixture was stirred overnight. The precipitated DCU was filtered through celite and the filtrate was evaporated to give a solid. The mixture of diastereoisomers were purified by flash chromatography $R_f$ = 0.30 (chloroform-acetone, 16:1) but could not be separated. The mixture was recrystallised four times from methanol to give a single pure diastereoisomer **(299)** (0.891g, 36.5%) in an unoptimised yield.

**(299)** m.p 212-214°C.
**(299)** $[\alpha]_D$ = +64° (c = 1 in $CH_2Cl_2$).
**(299)** (Found: C, 62.7; H, 5.88. $C_{32}H_{36}O_{12}$ requires C, 62.74; H, 5.92).

**(299)** $\delta_H$ ($CDCl_3$; 270MHz) 1.21, 1.29, 1.32, 1.57 (12H, 4s, C*Me*$_2$), 2.17, 2.18 (6H, 2s, Ins-$O_2$C-CH-(O*Ac*)Ph), 3.23 (1H, dd, J 9.34, 11.01, H-5, Ins), 4.04 (1H, t, J 10.45, H-4, Ins), 4.15 (1H, dd, J 4.77, 6.61, H-1, Ins), 4.54 (1H, t, J 4.58, H-2, Ins), 5.08 (1H, dd, J 4.22, 10.63, H-3, Ins), 5.22 (1H, dd, J 6.6, 10.99, H-6, Ins), 6.01 (1H, s, Ins-$O_2$C-C*H*-(OAc)Ph), 6.11 (1H, s, Ins-$O_2$C-C*H*-(OAc)Ph), 7.33-7.52 (10H, m, Ins-$O_2$C-CH-(OAc)*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 20.59, 20.72, 25.72, 26.56 (4q, C*Me*$_2$), 26.69, 27.73 (2q, Ins-$O_2$C-CH-(O*Ac*)Ph, -OC(O)*C*$H_3$), 71.34, 74.05, 74.34, 74.48, 75.04, 75.67, 76.37, 78.65 (8d, Ins-$O_2$C-*C*H-(OAc)Ph and 6 *C*H *myo*-inositol ring carbons), 110.72, 112.61 (2s, *C*$Me_2$), 127.92, 128.17, 128.54, 128.59, 129.07, 129.20 (6d, Ins-$O_2$C-CH-(OAc)*Ph*), 167.74, 168.40, 169.85, 170.54 (4s, Ins-$O_2$*C*-CH-(O*Ac*)Ph, -O*C*(O)$CH_3$).

m/z (+ve ion FAB) 613 [M + H, (50%)] 555 (30%) 149 (90%) 107 (100%).

### 6.3.2 D-1,2:4,5-Di-*O*-isopropylidene-*myo*-inositol (301)

A mixture of D-3,6-di-*O*-[*S*-(+)-*O*-acetylmandelyl]-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(299)** (0.74g, 1.21mmol), sodium hydroxide (0.40g, 10mmol) and methanol (100ml) was heated at reflux temperature for 30min. The mixture was cooled and neutralised with carbon dioxide. The solid was then diluted with water (50ml) and evaporated to dryness *in vacuo*. The crude product was extracted with dichloromethane (4x100ml) and the solvent was evaporated to give a white solid. The title compound **(301)** was purified by flash chromatography (ethyl acetate-dichloromethane, 1:1), $R_f$ = 0.20, (ether), dried over magnesium sulphate and evaporated to give the product. The solid was recrystallised from ethyl acetate to give **(301)**. Yield, (0.271g, 86%).

m.p. 174-176°C (from ethyl acetate); (lit. [478] 176-177°C).
$[\alpha]_D$ = -22° (c = 1 in acetonitrile); (lit. [478] -21.7°, c = 0.46 in acetonitrile).
(Found: C, 55.6; H, 7.88. $C_{12}H_{20}O_6$ requires C, 55.4; H, 7.75).

The NMR data were identical to **(37)**.

### 6.3.3 D-3,6-Di-*O*-benzyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol (302)

A mixture of D-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(301)** (0.209g, 0.80mmol), DMF (10ml) and sodium hydride (0.096g, 4mmol), was stirred at room temperature. Benzyl bromide (0.2ml, 2mmol) was added and stirred for a further 2h. TLC (ether-light petroleum, 1:1) then showed a new spot $R_f$ = 0.60. Methanol (2ml) was added to destroy the excess sodium hydride and the solvents were evaporated *in vacuo*. The residue was partitioned between water and ether (30ml each) and the organic layer was evaporated to give a solid. The title compound **(302)** was purified by flash chromatography (ether-pentane, 1:2) and recrystallised from hexane. Yield, (0.319g, 91%).

m.p. 157-159°C (from hexane); (lit. [475] 159-161°C, for L-enantiomer).
$[\alpha]_D$ = -44° (c = 1 in $CH_2Cl_2$); (lit. [475] +85°, c = 1 in $CHCl_3$, for L-enantiomer).
(Found: C, 71.1; H, 7.35. $C_{26}H_{32}O_6$ requires C, 70.89; H, 7.32).

The NMR data were identical to **(106)**.

### 6.3.4 D-3,6-Di-*O*-benzyl-*myo*-inositol (285)

A mixture of D-3,6-di-*O*-benzyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(302)** (0.274g, 0.62mmol) and methanol-1M HCl (50ml, 9:1), were heated at reflux temperature for 30min. The solution was cooled and the solvents were evaporated to give a solid which was recrystallised from ethanol, $R_f$ = 0.60 (chloroform-methanol, 6:1). Yield, (0.213g, 95%).

m.p. 172-173°C (from ethanol).
$[\alpha]_D$ = +16° (c = 1 in methanol).

The mass spectrum and NMR data were identical to **(285)** described previously.

## 6.4 Synthesis of 2,5-di-*O*-Methyl-*myo*-Inositol 1,3,4,6-Tetrakisphosphate, 2,5-Di-*O*-Methyl-*myo*-Inositol 1,3,4,6-Tetrakisphosphorothioate and *myo*-Inositol 1,3,4,6-Tetrakisphosphorothioate

### 6.4.1 DL-3,6-Di-*O*-allyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol (303)

The title compound was prepared by the method of Gigg and coworkers. [349]
A mixture of DL-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(37)** (18.2g, 70mmol), DMF (200ml) and sodium hydride (4.8g, 200mmol), was stirred at room temperature. Allyl bromide (17.3ml, 200mmol) was added dropwise over a 10min period and the mixture was stirred at room temperature for 3h. TLC (ether), showed a new product $R_f$ = 0.80 and excess allyl bromide ($R_f$ = 0.95). The reaction was cooled with ice and the excess sodium hydride was destroyed with methanol (20ml). The mixture was diluted with water (300ml) and the title compound extracted with ether (6x200ml). The ether solution was

dried over magnesium sulphate and evaporated to give a solid. The solid was purified by flash chromatography to give the pure title compound **(303)**. Yield, (20.50g, 86%).

m.p. 85-86°C (from pentane); (lit. [349] 85-86°C).

$\delta_H$ ($CDCl_3$; 270MHz) 1.38, 1.43, 1.45, 1.54 (12H, 4s, C*Me*$_2$), 3.35 (1H, dd, J 9.34, 10.62, H-5, Ins), 3.66 (1H, dd, J 6.6, 10.62, H-4, Ins), 3.80 (1H, dd, J 4.21, 10.26, H-3, Ins), 3.98 (1H, t, J 9.89, H-6, Ins), 4.09 (1H, dd, J 5.0, 6.41, H-1, Ins), 4.20-4.37 (4H, m, Ins-O-C*H*$_2$CH=CH$_2$), 4.47 (1H, t, J 4.5, H-2, Ins), 5.17-5.36 (4H, m, Ins-O-CH$_2$CH=C*H*$_2$), 5.87-6.05 (2H, m, Ins-O-CH$_2$C*H*=CH$_2$).

### 6.4.2 DL-1,4-Di-*O*-allyl-*myo*-inositol (304)

The title compound was prepared by the method of Gigg and coworkers. [485] DL-3,6-Di-*O*-allyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(303)** (17g, 50mmol) was dissolved in 80% acetic acid (200ml) and the mixture was heated under reflux for 30min. The mixture was then cooled and the acetic acid was evaporated *in vacuo* to give a solid. The solid was co-evaporated *in vacuo* with water (100ml), to remove any remaining acetic acid. The resulting solid was recrystallised from ethanol to give the title compound **(304)**. Yield, (10.92g, 84%).

m.p. 136-137°C (from ethanol); (lit. [485] 137-139°C).

$\delta_H$ ($d_6$-DMSO; 270MHz) 2.96 (1H, dd, J 2.38, 9.7, H-1, Ins), 3.01 (1H, dt, J 4.58, 8.79, H-5, Ins), 3.16-3.28 (2H, m, H-3 and H-4, Ins), 3.48 (1H, dt, J 4.76, 9.34, H-6, Ins), 3.85 (1H, br s, H-2, Ins), 3.97-4.28 (4H, m, Ins-O-C*H*$_2$CH=CH$_2$), 4.53 (1H, d, J 6.22, $D_2O$ ex, Ins-OH), 4.62 (1H, d, J 3.67, $D_2O$ ex, Ins-OH), 4.67 (1H, d, J 4.76, $D_2O$ ex, Ins-OH), 4.69 (1H, d, J 4.77, $D_2O$ ex, Ins-OH), 5.01-5.33 (4H, m, Ins-O-CH$_2$CH=C*H*$_2$), 5.83-6.00 (2H, m, Ins-O-CH$_2$C*H*=CH$_2$).

### 6.4.3 DL-1,4-Di-*O*-allyl-3,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol (306) and DL-1,4-di-*O*-allyl-3,5-di-*O*-*p*-methoxybenzyl-*myo*-inositol (305)

A mixture of DL-1,4-di-*O*-allyl-*myo*-inositol **(304)** (5.2g, 20mmol) and dibutyltin oxide (12.5g, 50mmol) was suspended in toluene (400ml) and heated under reflux for 2.5h whilst removing water in a Dean and Stark apparatus. The solution was cooled and the toluene was evaporated under reduced pressure. The crystalline tin complex was dried at 130°C under reduced pressure for a further 0.5h. DMF (150ml) and CsF (15.19g, 100mmol), were added to the tin complex and the suspension was stirred vigorously under nitrogen. *p*-Methoxybenzyl chloride (12.53g, 60mmol) was added dropwise and the reaction was monitored by TLC (ether). After 24h at room temperature, TLC showed two products, $R_f = 0.40$ and 0.56. The DMF solution was evaporated *in vacuo* and the residue was partitioned between a saturated aqueous solution of sodium hydrogen carbonate (300ml) and dichloromethane (300ml). The solution was stirred for 30min and the precipitated tin derivatives were filtered through celite. The celite was then washed with dichloromethane (2x100ml). The organic layer was separated and washed with water, saturated brine and water again (200ml of each). The dichloromethane solution was dried over magnesium sulphate and evaporated to give an oil. The mixture was purified by flash chromatography (ether-light petroleum, 2:1) to give the title compounds, **(306)**, (2.41g, 24%) and **(305)** (1.2g, 12%).

**(306)** m.p. 115-117°C (from ethyl acetate-hexane).
**(305)** m.p. 94-95°C (from ethyl acetate-hexane).
**(306)** (Found: C, 66.9; H, 7.28. $C_{28}H_{36}O_8$ requires C, 67.17; H, 7.25).
**(305)** (Found: C, 67.1; H, 7.20. $C_{28}H_{36}O_8$ requires C, 67.17; H, 7.25).

**(306)** $\delta_H$ ($CDCl_3$; 270MHz) 2.62 (2H, br s, $D_2O$ ex, Ins-OH), 3.24 (1H, dd, J 2.56, 9.52, H-3 or H-1, Ins), 3.30 (1H, dd, J 2.56, 9.52, H-3 or H-1, Ins), 3.41 (1H, t, J 9.34, H-5, Ins), 3.68 (1H, t, J 9.34, H-4 or H-6, Ins), 3.76 (1H, t, J 9.52, H-4 or H-6, Ins), 3.77 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.79 (3H, s, Ins-O-$CH_2$PhO*Me*), 4.04-4.42 (5H, m, Ins-O-$CH_2$CH=$CH_2$ and H-2, Ins), 4.58-4.85 (4H, m, Ins-O-$CH_2$PhOMe), 5.14-5.33 (4H, m, Ins-O-$CH_2$CH=$CH_2$), 5.87-6.05 (2H, m, Ins-O-$CH_2$*C*H=$CH_2$), 6.84-6.90 (4H, m, Ins-O-$CH_2$*Ph*OMe), 7.24-7.31 (4H, m, Ins-O-$CH_2$*Ph*OMe).

$\delta_C$ ($CDCl_3$; 68MHz) 55.2 (q, Ins-O-$CH_2$PhO*Me*), 71.61, 72.29, 74.30, 75.15 (4t, Ins-O-$CH_2$CH=$CH_2$ and Ins-O-$CH_2$PhOMe), 67.75, 74.21, 79.20, 79.40, 80.21, 80.27 (6d, *C*H, *myo*-inositol ring carbons), 113.8 (d, Ins-O-$CH_2$*Ph*OMe), 116.79, 117.38 (2t, Ins-O-$CH_2$CH=$CH_2$), 129.44, 129.64 (2d, Ins-O-$CH_2$*Ph*OMe), 130.00 (s, Cq, Ins-O-$CH_2$*Ph*OMe), 135.28, 134.67 (2d, Ins-O-$CH_2$*C*H=$CH_2$), 159.32 (s, Cq, Ins-O-$CH_2$*Ph*OMe).

m/z (-ve ion FAB) 653 [M + NBA, (35%)] 499 [M - H, (100%)] 379 [M - $CH_2$PhOMe, (30%)].

**(305)** $\delta_H$ ($CDCl_3$; 270MHz) 2.48 (1H, br s, $D_2O$ ex, Ins-OH), 2.57 (1H, br s, $D_2O$ ex, Ins-OH), 3.13 (1H, dd, J 2.75, 9.70, H-3 or H-1, Ins), 3.25 (1H, t, J 9.34, H-5, Ins), 3.33(1H, dd, J 2.74, 9.52, H-3 or H-1, Ins), 3.76 (1H, t, J 9.52, H-4 or H-6, Ins), 3.79 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.80 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.94 (1H, t, J 9.52, H-4 or H-6, Ins), 4.06-4.43 (5H, m, Ins-O-C$H_2$CH=$CH_2$ and H-2 Ins), 4.60-4.85 (4H, m, Ins-O-C$H_2$PhOMe), 5.16-5.34 (4H, m, Ins-O-$CH_2$CH=C$H_2$), 5.87-6.05 (2H, m, Ins-O-$CH_2$C*H*=$CH_2$), 6.85-6.90 (4H, m, Ins-O-$CH_2$*Ph*OMe), 7.25-7.32 (4H, m, Ins-O-$CH_2$*Ph*OMe).

$\delta_C$ ($CDCl_3$; 68MHz) 55.23 (q, Ins-O-$CH_2$PhO*Me*), 75.08, 74.43, 72.42, 71.26 (4t, Ins-O-*C*$H_2$CH=$CH_2$ and Ins-O-*C*$H_2$PhOMe), 67.17, 72.00, 78.78, 79.46, 80.56, 82.41 (6d, *C*H, *myo*-inositol ring carbons), 113.84 (d, Ins-O-$CH_2$*Ph*OMe), 116.57, 117.74 (2t, Ins-O-$CH_2$CH=*C*$H_2$), 129.48, 129.61 (2d, Ins-O-$CH_2$*Ph*OMe), 130.06, 130.84 (2s, Cq, Ins-O-$CH_2$*Ph*OMe), 134.50, 135.28 (2d, Ins-O-$CH_2$*C*H=$CH_2$), 159.32, 159.32 (2s, Cq, Ins-O-$CH_2$*Ph*OMe).

m/z (-ve ion FAB) 653 [M + NBA, (100%)] 499 [M - H (35%)] 460 [M - $CH_2$CH=$CH_2$, (20%)] 379 [M - $CH_2$PhOMe (20%)] 335 (20%) 123 (40%).

### 6.4.4 DL-1,4-Di-*O*-allyl-3,6-di-*O*-*p*-methoxybenzyl-2,5-di-*O*-methyl-*myo*-inositol (307)

A mixture of sodium hydride (0.48g, 20mmol) and DL-1,4-di-*O*-allyl-3,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol **(306)** (2.0g, 4mmol) was stirred in DMF (50ml) at room temperature. Methyl iodide (1.25ml, 20mmol) was added dropwise and the mixture was stirred for 3h. TLC (ether), showed one spot $R_f$ = 0.80. The excess sodium hydride was destroyed with methanol (10ml) and the DMF solution was evaporated *in vacuo*. The product was taken up in ether (100ml), washed with water (50ml), brine (50ml) and water again (50ml). The organic layer was dried over magnesium sulphate, evaporated and purified by flash chromatography (ether-hexane, 2:1). The resulting syrup was recrystallised from hexane to give **(307)**. Yield, (1.91g, 90%).

m.p. 93-94°C (from hexane).
(Found: C, 68.4; H, 7.73. $C_{30}H_{40}O_8$ requires C, 68.15; H, 7.73).

$\delta_H$ ($CDCl_3$; 270MHz) 3.04 (1H, t, J 9.25, H-5, Ins), 3.14 (1H, dd, J 2.38, 9.89, H-3 or H-1, Ins), 3.33 (1H, dd, J 2.20, 9.89, H-3 or H-1, Ins), 3.60 (3H, s, Ins-O*Me*), 3.64 (3H, s, Ins-O*Me*), 3.66-3.73 (3H, m, H-2, H-4, H-6, Ins), 3.78 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.79 (3H, s, Ins-O-$CH_2$PhO*Me*), 4.04-4.39 (4H, m, Ins-O-$CH_2$CH=$CH_2$), 4.40-4.85 (4H, m, Ins-O-C*$H_2$*PhOMe), 5.14-5.32 (4H, m, Ins-O-$CH_2$CH=C*$H_2$*), 5.85-6.07 (2H, m, Ins-O-$CH_2$C*H*=$CH_2$), 6.85-6.90 (4H, m, Ins-O-$CH_2$*Ph*OMe), 7.25-7.32 (4H, m, Ins-O-$CH_2$*Ph*OMe).

$\delta_C$ ($CDCl_3$; 68MHz) 55.10 (q, Ins-O-$CH_2$PhO*Me*), 61.05, 61.20 (2q, Ins-O*Me*), 71.76, 72.58, 74.27, 75.33 (4t, Ins-O-*C*$H_2$CH=$CH_2$ and Ins-O-*C*$H_2$PhOMe), 77.53, 80.04, 80.14, 81.21, 81.24, 85.36 (6d, *C*H, *myo*-inositol ring carbons), 113.61 (d, Ins-O-$CH_2$*Ph*OMe), 116.34, 116.73 (2t, Ins-O-$CH_2$CH=*C*$H_2$), 129.15, 129.65 (2d, Ins-O-$CH_2$*Ph*OMe), 130.40, 131.07 (2s, Cq, Ins-O-$CH_2$*Ph*OMe), 134.44, 135.89 (2d, Ins-O-$CH_2$*C*H=$CH_2$), 159.04, 159.09 (2s, Cq, Ins-O-$CH_2$*Ph*OMe).

m/z (-ve ion FAB) 681 [M + NBA, (60%)] 513 [M - Me, (60%)] 485 (43%) 440 (52%) 335 (50%) 276 (45%) 258 (48%) 154 (100%) 120 (62%).

### 6.4.5 2,5-Di-*O*-methyl-*myo*-inositol (308)

A mixture of DL-1,4-di-*O*-allyl-3,6-di-*O*-*p*-methoxybenzyl-2,5-di-*O*-methyl-*myo*-inositol **(307)** (1.056g, 2mmol) and palladium on activated charcoal, (10% Fluka, 0.30g) and toluene-*p*-sulphonic acid (0.10g, 0.52mmol) was dissolved in a mixture of ethanol (55ml) and water (5ml) and refluxed for 24h. TLC (chloroform-methanol 3:1), showed a single product $R_f$ = 0.2. The solution was filtered through celite and recrystation from ethanol gave the title compound **(306)**. Yield, (0.321g, 77%).

m.p. 266-268°C (from ethanol); (lit. [377] 270°C).
(Found: C, 46.1; H, 7.70. $C_8H_{16}O_6$ requires C, 46.13; H, 7.75).

$\delta_H$ ($d_6$-DMSO; 270MHz) 2.65 (1H, t, J 9.0, H-5, Ins), 3.19 (2H, ddd, J 2.56, 5.31, 9.89, H-3 and H-1, Ins), 3.35 (1H, br s, H-2, Ins), 3.36 (2H, dt, J 4.95, 10.1, H-4 and H-6, Ins), 3.44 (6H, s, Ins-O*Me*), 4.57 (2H, d, J 5.13, $D_2O$ ex, -OH), 4.67 (2H, d, J 4.76, $D_2O$ ex, -OH).

$\delta_C$ ($d_6$-DMSO; 68MHz) 72.16, 72.58, 83.48, 85.72 (4d, *C*H, *myo*-inositol ring carbons), 51.29, 59.90 (2q, Ins-O*Me*).

### 6.4.6 DL-1,4-Di-*O*-allyl-2,5-di-*O*-benzyl-3,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol (309)

A mixture of DL-1,4-di-*O*-allyl-3,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol **(306)** (2.0g, 4mmol) and sodium hydride (480mg, 20mmol) was stirred in dry DMF (20ml). Benzyl bromide (1.19ml, 10mmol) was added dropwise to the stirred solution at room temperature. After 2h TLC (ether-hexane, 2:1) showed one spot, $R_f$ = 0.60. Methanol (5ml), was added to destroy the excess sodium hydride and the solvent was evaporated *in vacuo*. The resulting syrup was partitioned between water and ether, washed with brine then water (100ml of each). The organic layer was dried over magnesium sulphate, filtered and evaporated to give a syrup. Flash chromatography (ether-hexane, 2:1) removed the remaining benzyl bromide to give the title compound **(309)**. Yield, (2.94g, 92%).

m.p. 91-92°C (from hexane).
(Found: C, 74.3; H, 7.04. $C_{42}H_{48}O_8$ requires C, 74.07; H, 7.11).

$\delta_H$ ($CDCl_3$; 270MHz) 3.21 (1H, dd, J 2.00, 9.90, H-3 or H-1, Ins), 3.27 (1H, dd, J 2.00, 9.90, H-3 or H-1, Ins), 3.37 (1H, t, J 9.34, H-5, Ins), 3.77 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.79 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.90 (1H, t, J 9.34, H-4 or H-6, Ins), 3.96 (1H, t, J 9.89, H-4 or H-6, Ins), 3.97 (1H, br s, H-2, Ins), 4.00-4.12 (2H, m, Ins-O-$CH_2$CH=$CH_2$), 4.27-4.43 (2H, m, Ins-O-$CH_2$CH=$CH_2$), 4.53, 4.59 (2H, AB, J 11.36, Ins-O-$CH_2$PhOMe or Ins-O-$CH_2$Ph), 4.71, 4.80 (2H, AB, J 10.26, Ins-O-$CH_2$PhOMe or Ins-O-$CH_2$Ph), 4.84 (4H, br s, Ins-O-$CH_2$PhOMe and/or Ins-O-$CH_2$Ph), 5.12-5.33 (4H, m, Ins-O-$CH_2$CH=$CH_2$), 5.84-6.03 (2H, m, Ins-O-$CH_2$*C*H=$CH_2$), 6.81 (2H, d, J 8.6, Ins-O-$CH_2$*Ph*OMe), 6.86 (2H, d, J 8.6, Ins-O-$CH_2$*Ph*OMe), 7.21-7.41 (14H, m, Ins-O-$CH_2$*Ph*OMe and Ins-O-$CH_2$*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 55.23 (q, Ins-O-$CH_2$PhO*Me*), 71.58, 72.52, 73.95, 74.53, 75.44, 75.86 (6t, Ins-O-$CH_2$CH=$CH_2$, Ins-O-$CH_2$PhOMe and Ins-O-$CH_2$Ph), 74.44, 80.50, 80.66, 81.28, 81.44, 83.65 (6d, *C*H, *myo*-inositol ring carbons), 113.71 (d, Ins-O-$CH_2$*Ph*OMe), 116.57 (t, Ins-O-$CH_2$CH=$CH_2$), 127.24, 127.47, 127.76, 127.86, 128.08, 128.31, 129.09, 129.77 (8d, Ins-O-$CH_2$*Ph*OMe and Ins-O-$CH_2$*Ph*), 130.61, 131.13 (2s, Cq, Ins-O-$CH_2$*Ph*OMe), 134.99, 135.51 (2d, Ins-O-$CH_2$*C*H=$CH_2$), 138.95, 139.01 (2s, Cq, Ins-O-$CH_2$*Ph*), 158.12 (s, Cq, Ins-O-$CH_2$*Ph*OMe).

m/z (-ve ion FAB) 833 [M + NBA, (50%)] 665 (50%) 605 (50%) 322 (55%) 302 (60%) 273 (100%) 118 (90%).

### 6.4.7 DL-2,5-Di-*O*-benzyl-3,6-di-*O*-*p*-methoxybenzyl-1,4-di-*O*-*cis*-prop-1-enyl-*myo*-inositol (310)

A mixture of DL-1,4-di-*O*-allyl-2,5-di-*O*-benzyl-3,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol **(309)** (2.38g, 3.38mmol) and sublimed potassium *t*-butoxide (1.57g, 14mmol) in dry DMF (40ml), was stirred at 85°C for 2h under an atmosphere of nitrogen. TLC (ether-hexane, 2:1), showed one spot, $R_f$ = 0.60, which was the same as starting material. The reaction was then cooled, water (100ml), was added and the product extracted with dichloromethane (4x100ml). The organic layer was dried over magnesium sulphate, filtered and purified by flash chromatography, (ether-hexane, 2:1), to give the title compound **(310)**. Yield, (1.90g, 83%).

m.p. 108-110°C (from hexane).
(Found: C, 74.3; H, 7.07. $C_{42}H_{48}O_8$ requires C, 74.07; H, 7.11).

$\delta_H$ ($CDCl_3$; 270MHz) 1.64 (3H, dd, J 1.47, 6.96, Ins-O-CH=CHC*H*$_3$), 1.66 (3H, dd, J 1.65, 7.14, Ins-O-CH=CHC*H*$_3$), 3.32 (1H, dd, J 2.57, 9.71, H-3 or H-1, Ins), 3.42 (1H, t, J 9.34, H-5, Ins), 3.51 (1H, dd, J 2.20, 9.70, H-3 or H-1, Ins), 3.77 (3H, s, Ins-O-CH$_2$PhO*Me*), 3.79 (3H, s, Ins-O-CH$_2$PhO*Me*), 3.98 (1H, br s, H-2, Ins), 4.02 (1H, t, J 9.53, H-4 or H-6, Ins), 4.14 (1H, t, J 9.89, H-4 or H-6, Ins), 4.35 (1H, dq, J 6.76, Ins-O-CH=C*H*CH$_3$), 4.44 (1H, dq, J 6.76, Ins-O-CH=C*H*CH$_3$), 4.51-4.82 (8H, m, Ins-O-C*H*$_2$PhOMe and Ins-O-C*H*$_2$Ph), 6.08 (1H, dd, J 1.65, 6.23, Ins-O-C*H*=CHCH$_3$), 6.26 (1H, dd, J 1.65, 6.41, Ins-O-C*H*=CHCH$_3$), 6.84-6.90 (4H, m, Ins-O-CH$_2$*Ph*OMe), 7.22-7.41 (14H, m, Ins-O-CH$_2$*Ph*OMe and Ins-O-CH$_2$*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 9.33, 9.40 (2q, Ins-O-CH=CH*C*H$_3$), 55.23 (q, Ins-O-CH$_2$PhO*Me*), 72.29, 74.46, 75.30, 75.73 (4t, Ins-O-*C*H$_2$PhOMe and Ins-O-*C*H$_2$Ph), 75.99, 78.42, 80.36, 82.57, 82.92, 84.39 (6d, *C*H, *myo*-inositol ring carbons), 98.14, 100.77 (2d, Ins-O-CH=*C*HCH$_3$), 113.71 (d, Ins-O-CH$_2$*Ph*OMe), 127.36, 127.59, 127.82, 128.11, 128.27, 129.24, 129.96 (7d, Ins-O-CH$_2$*Ph*OMe and Ins-O-CH$_2$*Ph*), 130.35, 130.80 (2s, Cq, Ins-O-CH$_2$*Ph*OMe), 138.59, 138.75 (2s, Cq, Ins-O-CH$_2$*Ph*), 145.75, 147.77 (2d, Ins-O-*C*H=CHCH$_3$), 159.18 (s, Cq, Ins-O-CH$_2$*Ph*OMe).

m/z (-ve ion FAB) 833 [M + NBA, (100%)] 559 [M - *p*-methoxybenzyl, (15%)] 470 (20%) 336 (20%) 272 (45%) 182 (60%).

### 6.4.8 2,5-Di-*O*-benzyl-*myo*-inositol (311)

2,5-Di-*O*-benzyl-3,6-di-*O*-*p*-methoxybenzyl-1,4-di-*O*-*cis*-prop-1-enyl-*myo*-inositol **(310)** (0.42g, 0.62mmol), was stirred in a mixture of dichloromethane-trifluoroacetic acid [20ml, (10:1)] at room temperature overnight. TLC (ether), showed the presence of a *p*-methoxybenzyl derivative only, and the orange-red solution was evaporated to dryness. The mixture was coevaporated with water then ethanol (10ml of each) to remove traces of acid, after which a fine white solid precipitated from the solution. The solid was filtered and washed successively with water, acetone and finally ether (20ml of each) to give **(311)**. Yield, (0.17g, 76%).

m.p. 271-273°C (from DMF-ethanol); (lit. [387] 270-272°C).
(Found: C, 66.4; H, 6.64. $C_{20}H_{24}O_6$ requires C, 66.67; H, 6.67).

$\delta_H$ ($d_6$-DMSO; 270MHz) 3.03 (1H, t, J 9.16, H-5, Ins), 3.33 (2H, ddd, J 2.57, 4.77, 9.71, H-3 and H-1, Ins), 3.60 (2H, dt, J 5.13, 9.34, H-4 and H-6, Ins), 3.73 (1H, t, J 2.75, H-2, Ins), 4.75 (2H, d, J 4.77, $D_2O$ ex, Ins-OH-1 and Ins-OH-3), 4.78 (4H, s, Ins-O-C$H_2$Ph), 4.83 (2H, d, J 5.12 $D_2O$ ex, Ins-OH-4 and Ins-OH-6), 7.20-7.43 (10H, m, Ins-O-$CH_2$*Ph*).

$\delta_C$ ($d_6$-DMSO; 68MHz) 73.75, 74.17 (2t, Ins-O-*C*$H_2$Ph), 72.19, 73.07, 81.83, 84.23 (4d, *C*H, *myo*-inositol ring carbons), 127.01, 127.53, 127.92, 127.98 (4d, Ins-O-$CH_2$*Ph*), 139.92, 140.01 (2s, Cq, Ins-O-$CH_2$*Ph*).

### 6.4.9 2,5-Di-*O*-methyl-1,3,4,6-tetrakis(diethoxyphospho)-*myo*-inositol (313)

A mixture of 2,5-di-*O*-methyl-*myo*-inositol **(308)** (0.104g, 0.5mmol) and dry diisopropylethylamine (0.7ml, 4mmol) was dissolved in dry DMF (5ml) and kept under nitrogen at -78°C. Diethoxychlorophosphine **(273)** (0.58ml, 4mmol), (90-95%) was added dropwise to the stirred solution and left for 45min. The mixture was oxidised with *t*-butylhydroperoxide (1ml, 7.3mmol) at -78°C and stirred for a further 30min at room temperature. The DMF was evaporated *in vacuo* and the remaining syrup was partitioned between water (50ml) and dichloromethane (50ml). The organic layer was washed with

10% sodium metabisulphite solution (20ml), brine (20ml) and finally water (2x20ml). The organic layer was dried over magnesium sulphate and evaporated to give **(313)** $R_f$ = 0.40 (chloroform-methanol, 3:1). Yield, (0.286g, 76%).

$\delta_H$ ($CDCl_3$; 270MHz) 1.26-1.39 (24H, m, Ins-O-P(O)O$CH_2$$CH_3$), 3.23 (1H, t, J 9.43, H-5, Ins), 3.57 (3H, s, Ins-O*Me*), 3.63 (3H, s, Ins-O*Me*), 4.12-4.27 (18H, Ins-O-P(O)O$CH_2$$CH_3$, H-3 and H-1, Ins), 4.40 (1H, br s, H-2, Ins), 4.74 (2H, q, J 9.46, H-4 and H-6, Ins).

$\delta_C$ ($CDCl_3$; 68MHz) 16.05, 16.15 (2q, Ins-O-P(O)O$CH_2$$CH_3$), 59.64, 61.78 (2q, Ins-O*Me*), 63.89, 63.99, 64.09, 64.22, 64.31, 64.44, 64.51 (7t, Ins-O-P(O)O$CH_2$$CH_3$), 75.70, 76.28, 76.41, 76.51, 78.00, 80.79 (6d, *C*H, *myo*-inositol ring carbons).

$\delta_P$ ($CDCl_3$; 109MHz) -2.63 (10H, q, J 7.47, -CH-O-*P*(O)O$CH_2$$CH_3$), -3.15 (10H, q, J 7.46 -CH-O-*P*(O)O$CH_2$$CH_3$).

m/z (+ve ion FAB) 753 [M + H, (100%)] 725 [M - 2Me + H, (18%)] 291 (48%) 235 (38%) 130 (32%).

Accurate mass spectrum requires: $(M + H)^+$ = 753.2182. Found 753.2231.

### 6.4.10 2,5-Di-*O*-methyl-*myo*-inositol 1,3,4,6-tetrakisphosphate (314)

2,5-Di-*O*-methyl-1,3,4,6-tetrakis(diethoxyphospho)-*myo*-inositol **(313)** (0.25g, 0.33mmol), was dissolved in dry dichloromethane (5ml) and kept under a blanket of nitrogen at room temperature. Trimethylsilyl bromide (0.703ml, 5.3mmol), was added dropwise and the solution was stirred for 16h at room temperature. Evaporation gave a residue which was dissolved in water (5ml), then stirred for 1h to give the title compound **(314)** in quantitative yield by $^{31}P$ NMR. A small portion was purified by ion exchange chromatography, using a buffer gradient of 200-1000mmol of TEAB and a flow rate of 5ml/min. The amount of product was determined by a quantitative Briggs test. Yield, (43.5µmol) which eluted at 500mmol buffer.

$\delta_H$ ($D_2O$; 270MHz) 3.51 (3H, br s, Ins-O*Me*), 3.57 (4H, br s, Ins-O*Me* and H-5, Ins), 3.97 (1H, br s. H-2, Ins), 4.06 (2H, t, J 9.52, H-3 and H-1, Ins), 4.28 (2H, q, J 9.40, H-4 and H-6, Ins).

$\delta_C$ ($D_2O$; 68MHz) 59.64, 61.39 (2q, Ins-O*Me*), 73.97, 75.66, 75.76, 76.21, 80.01, 81.60 (6d, *C*H, *myo*-inositol ring carbons).

$\delta_P$ ($D_2O$; 162MHz) 0.00 (1H, d, J 9.9, -CH-O-$PO_3^{2-}$), -0.58 (1H, d, J 8.0, -CH-O-$PO_3^{2-}$).

m/z (-ve ion FAB) 527 [M - H, (100%)] 447 (35%) 250 (40%) 97 (64%).

Accurate mass spectrum requires: $(M - H)^-$ = 526.9520. Found 526.9520.

### 6.4.11 2,5-Di-*O*-methyl-1,3,4,6-tetrakis[di(benzyloxyphosphorothio)]-*myo*-inositol (316)

A mixture of bis(benzyloxy)diisopropylaminophosphine **(96)** (0.69g, 2mmol) and 1*H*-tetrazole (350mg, 5mmol) was stirred in DMF (5ml) for 1h. 2,5-Di-*O*-methyl-*myo*-inositol **(308)** (0.052g, 0.25mmol), was added to the mixture which was stirred for a further 2h. TLC (ether-petroleum ether, 2:1), showed a major product $R_f$ = 1.00 for the phosphite. Sulphur (0.096g, 3mmol) and dry pyridine (2ml) were added and the mixture stirred for 15min after which TLC (ether-petroleum ether, 2:1), showed a new spot $R_f$ = 0.60. The excess sulphur was filtered and the solvents were evaporated *in vacuo* to give a syrup. The title compound **(316)** was purified by flash chromatography (ether-petroleum ether, 2:1) and isolated as a syrup. Yield, (0.27g, 82%).

(Found: C, 58.3; H, 5.21. $C_{64}H_{68}O_{14}P_4S_4$ requires C, 58.53; H, 5.22).

$\delta_H$ ($CDCl_3$; 270MHz) 2.23 (1H, t, J 9.34, H-5, Ins), 3.31 (3H, s, Ins-O*Me*), 3.50 (3H, s, Ins-O*Me*), 4.33 (2H, dt, 2.02, 9.89, H-3 and H-1, Ins), 4.61 (1H, br s, H-2, Ins), 5.00-5.14 (18H, m, Ins-O-P(S)O-C*H*$_2$Ph, H-4 and H-6, Ins), 7.20-7.37 (40H, m, Ins-O-P(S)O-CH$_2$*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 61.29, 61.33 (2q, Ins-O*Me*), 69.47, 70.09 (2t, Ins-O-P(S)O-*C*H$_2$Ph), 76.02, 77.00, 77.25, 79.10, 79.30, 80.66 (6d, *C*H, *myo*-inositol ring carbons), 127.69, 127.82, 127.89, 127.95, 128.08, 128.24 (6d, Ins-O-P(S)O-CH$_2$*Ph*), 135.18, 135.28, 135.41, 135.48, 135.57, 135.67, 135.80 (7s, Ins-O-P(S)O-CH$_2$*Ph*, Cq).

$\delta_P$ ($CDCl_3$; 162MHz) +69.11 (dtt, J 8.0, 9.9, 9.9, -C*H*-O-*P*(S)OC*H*$_2$Ph), +67.09 (dtt, J 7.9, 9.9, 9.9, -C*H*-O-*P*(S)OC*H*$_2$Ph).

m/z (+ve ion FAB) 1314 [M + H, (0.15%)] 725 (0.1%) 430 (0.48%) 181 (10%) 91 (100%).

### 6.4.12 2,5-Di-*O*-methyl-*myo*-inositol 1,3,4,6-tetrakisphosphorothioate (317)

Ammonia (80ml), was distilled into a three neck flask and small slithers of freshly cut sodium metal (0.80g, 34.8mmol), were added until the solution remained blue. The dry-ice condenser was moved across to the reaction flask and ammonia (40ml), was gently transferred to the flask by heating. Small slithers of sodium (0.40g, 17.4mmol), were added to the ammonia until the colour remained blue. 2,5-Di-*O*-methyl-1,3,4,6-tetrakis[di(benzylphosphorothio)]-*myo*-inositol **(316)**, (0.10g, 76μmol), in dry dioxan (1ml) was added to the sodium in liquid ammonia. The reaction was left for 2min and quenched with methanol (20ml). The ammonia was evaporated in a stream of nitrogen, MilliQ water was then added to the residue which was evaporated to dryness *in vacuo*. The deprotected phosphorothioate **(317)** was purified by ion exchange chromatography using a buffer gradient of 0-1000mmol and eluted at *ca*. 800mmol. Yield, (19.2μmol, 25%).

$\delta_H$ ($D_2O$; 270MHz) 3.32 (1H, t, J 9.52, H-5, Ins), 3.60 (3H, s, Ins-O*Me*), 3.64 (3H, s, Ins-O*Me*), 4.15 (1H, t, J 2.38, H-2, Ins), 4.23 (2H, ddd, J 2.3, 10.26, 10.26, H-3 and H-1, Ins), 4.60 (2H, q, J 10.00, H-4 and H-6, Ins).

$\delta_C$ ($D_2O$; 68MHz) 59.57, 61.65 (2q, Ins-O*Me*), 74.07, 76.44, 80.14, 82.02 (4d, *C*H, *myo*-inositol ring carbons).

$\delta_P$ ($D_2O$; 109MHz) +46.7 (d, J 10.1), +48.8 (d, J 10.1).

m/z (-ve ion FAB) 591 [M - H, (100%)] 557 (50%) 460 (62%) 308 (48%) 175 (50%) 95 (95%).

Accurate mass spectrum requires: $(M - H)^-$ = 590.8662. Found 590.8608.

### 6.4.13 2,5-Di-*O*-benzyl-1,3,4,6-tetrakis[di(benzyloxyphosphorothio)]-*myo*-inositol (319)

A mixture of bis(benzyloxy)diisopropylaminophosphine (**96**) (0.69g, 2mmol) and 1*H*-tetrazole (0.35g, 5mmol) in DMF (2ml) was stirred for 1h. 2,5-Di-*O*-benzyl-*myo*-inositol **(311)** (0.90g, 0.25mmol), was added to the mixture and stirred for a further 1h. TLC (ether-petroleum ether, 1:2), showed a major product $R_f = 1.00$ for the phosphite. Sulphur (0.096g, 3mmol) and dry pyridine (2ml) were added and stirred for 10min after which TLC (ether-petroleum ether, 1:2), showed a new spot $R_f = 0.40$. The excess sulphur was filtered and the solvents were evaporated at room temperature *in vacuo*. The title compound was purified by flash chromatography (ether-petroleum ether, 1:2) and isolated as a syrup. Yield, (0.26g, 71%).

(Found: C, 62.5; H, 5.32. $C_{76}H_{76}O_{14}P_4S_4$ requires C, 62.29; H, 5.23).

$\delta_H$ ($CDCl_3$; 400MHz) 3.59 (1H, t, J 9.15, H-5, Ins), 4.42-4.48 (3H, m, H-1, H-2 and H-3, Ins), 4.78-5.07 (20H, m, Ins-O-P(S)O-C$H_2$Ph and Ins-O-C$H_2$Ph), 5.38 (2H, dt, J 9.15, 12.52, H-4 and H-6, Ins), 6.86-7.37 (50H, Ins-O-P(S)O-$CH_2$*Ph* and Ins-O-$CH_2$*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 69.99, 70.19, 70.25, 70.54, 70.74, 74.01, 75.83 (7t, Ins-O-P(S)O-*C*$H_2$Ph, Ins-O-*C*$H_2$Ph), 76.51, 76.93, 77.84, 79.30 (4d, *C*H, *myo*-inositol ring carbons), 127.01, 127.27, 127.53, 127.66, 128.21, 128.37, 128.57, 128.63, 128.73, 128.80, 128.86, 128.93 (12d, Ins-O-P(S)O-$CH_2$*Ph* and Ins-O-$CH_2$*Ph*), 135.74, 135.87, 135.93, 136.06, 136.13, 136.23, 136.36, 136.49, 138.75, 138.85 (10s, Cq, Ins-O-P(S)O-$CH_2$*Ph* and Ins-O-$CH_2$*Ph*).

$\delta_P$ ($CDCl_3$; 109MHz) +66.78 (dtt, J 9.34, 9.52), +69.57 (dtt, J 9.71, 11.44).

m/z (-ve ion FAB) 591 [M - Bn, (5.5%)] 1281 (1%) 293 (100%) 95 (30%).

### 6.4.14 *myo*-Inositol 1,3,4,6-tetrakisphosphorothioate (320)

Ammonia (80ml), was distilled into a three neck flask and small slithers of freshly cut sodium metal (800mg, 34.8mmol), were added until the solution remained blue. The dry-ice condenser was moved across to the reaction flask and ammonia (40ml), was transferred to the flask by heating. Small slithers of sodium (400mg, 17.4mmol), were added to the ammonia until the colour remained blue. A solution of 2,5-di-*O*-benzyl-1,3,4,6-tetrakis[di(benzylphosphorothio)]-*myo*-inositol **(319)**, (0.059g, 76μmol) in dry dioxan (1ml), was added to the sodium in liquid ammonia. The reaction was left for 2min and quenched with methanol (20ml). The ammonia was evaporated in a stream of nitrogen, MilliQ water was then added to the residue which was evaporated to dryness *in vacuo*. The deprotected phosphorothioate **(320)** was purified by ion exchange chromatography using a buffer gradient of 0-1000mmol and eluted at *ca.* 800mmol. Yield, (18.5μmol, 46%).

$\delta_H$ ($D_2O$; 270MHz) 3.61 (1H, t, J 8.98, H-5, Ins), 4.16 (1H, dt, J 2.95, 9.52, H-1 and H-3, Ins), 4.51 (2H, q, J 9.89, H-4 and H-6, Ins), 4.99 (1H, br s, H-2, Ins).

$\delta_P$ ($D_2O$; 109MHz) +42.4 (d, J 12.25), +45.09 (d, J 9.77).

m/z (-ve ion FAB) 563 [M - H, (100%)] 529 (15%) 232 (20%) 95 (95%).

Accurate mass spectrum requires: $(M - H)^-$ = 562.8295 Found 562.8314.

## 6.5 Synthesis of DL-*myo*-Inositol 1,4,6-Trisphosphate and DL-*myo*-Inositol 1,4,6-Trisphosphorothioate

### 6.5.1 DL-1,4-Di-*O*-allyl-2,3-*O*-isopropylidene-*myo*-inositol (321)

A mixture of DL-3,6-di-*O*-allyl-1,2:4,5-di-*O*-isopropylidene-*myo*-inositol **(301)** (10.7g, 31.47mmol) toluene-*p*-sulphonic acid (0.10g, 0.5mmol) and ethane 1,2-diol (1.75ml, 31.47mmol), in dichloromethane (100ml), was stirred at 0°C in an ice bath for 10min. The ice bath was removed and the mixture was stirred for a further 30min until the solution became slightly cloudy. Triethylamine (2ml), was added to the cloudy solution followed by water (100ml). The starting material and product were separated leaving DL-1,4-di-*O*-allyl-*myo*-inositol in the water layer. The product $R_f$ = 0.30 (ether), was separated and the organic layer was evaporated to give the title compound and starting material which was recrystallised from ethyl acetate-hexane to give the pure title compound **(321)**. Yield, (6.4g, 68%).

m.p. 130°C; (lit. [349] 130-132°C).

$\delta_H$ ($CDCl_3$; 270MHz) 1.37, 1.54 (6H, 2s, C*Me*$_2$), 3.10 (2H, s, $D_2O$ ex, Ins-OH), 3.36 (1H, t, J 9.53, H-5, Ins), 3.49 (1H, t, J 9.53, H-6, Ins), 3.49 (1H, dd, J 2.56, 9.53, H-3, Ins), 3.89 (1H, t, J 9.34, H-4, Ins), 4.08 (1H, dd, J 5.31, 6.78, H-1, Ins), 4.17-4.39 (4H, m, Ins-O-C*H*$_2$CH=CH$_2$), 4.43 (1H, dd, J 3.97, 5.31, H-2, Ins), 5.16-5.36 (4H, m, Ins-O-CH$_2$CH=C*H*$_2$), 5.87-6.05 (2H, m, Ins-O-CH$_2$C*H*=CH$_2$).

$\delta_C$ ($CDCl_3$; 68MHz) 25.91, 27.99 (2q, C*Me*$_2$), 71.64, 72.29 (2t, Ins-O-*C*H$_2$CH=CH$_2$), 71.38, 72.94, 73.88, 76.80, 79.20, 81.63 (6d, *C*H, *myo*-inositol ring carbons), 109.88 (s, Cq, *C*Me$_2$), 117.38, 118.06 (2t, Ins-O-CH$_2$CH=*C*H$_2$), 134.60, 134.73 (2d, Ins-O-CH$_2$*C*H=CH$_2$).

### 6.5.2 Selective alkylation of 3,6-di-*O*-allyl-1,2-*O*-isopropylidene-*myo*-inositol (using *p*-methoxybenzyl chloride) (322, 323, 324)

A mixture of DL-1,4-di-*O*-allyl-2,3-*O*-isopropylidene-*myo*-inositol **(321)** (4.5g, 15mmol), acetonitrile (300ml), dibutyltin oxide (5g, 20mmol), tetrabutylammonium iodide (7.38g, 20mmol) and *p*-methoxybenzyl chloride (5.42ml, 40mmol) was heated under reflux in a Soxhlet apparatus containing molecular sieves (4Å, 30g), for 48h.

The reaction mixture was cooled, the solvent was evaporated and the orange residue was partitioned between water (250ml) and ether (250ml). The organic layer was separated and stirred with a saturated solution of sodium hydrogen carbonate (250ml), for 1h. The solid was removed by filtration through celite, washed with ether and the organic layer was dried over magnesium sulphate. TLC (ether-hexane, 3:2) showed five spots. *p*-Methoxybenzyl iodide, $R_f = 0.70$; *p*-methoxybenzyl chloride, $R_f = 0.60$; DL-1,4-di-*O*-allyl-2,3-*O*-isopropylidene-5,6-di-*O*-*p*-methoxybenzyl-*myo*-inositol, $R_f = 0.50$ **(322)**; DL-1,4-di-*O*-allyl-2,3-*O*-isopropylidene-6-*O*-*p*-methoxybenzyl-*myo*-inositol, $R_f = 0.40$ **(324)** and DL-1,4-di-*O*-allyl-2,3-*O*-isopropylidene-5-*O*-*p*-methoxybenzyl-*myo*-inositol, $R_f = 0.22$ **(323)** which were separated by flash chromatography, to give the products as syrups. DL-1,4-Di-*O*-allyl-2,3-*O*-isopropylidene-5-*O*-*p*-methoxybenzyl-*myo*-inositol, **(323)** was then recrystallised from hexane. Yields, **(322)** (1.15g, 14%), **(324)** (3.27g, 52%), **(323)** (1.96g, 31%).

**(323)** m.p. 70-72°C (from hexane).
**(322)** (Found: C, 69.0; H, 7.54. $C_{31}H_{40}O_8$ requires C, 68.85; H,7.46 ).
**(323)** (Found: C, 65.7; H, 7.79. $C_{23}H_{33}O_7$ requires C, 65.68; H, 7.68).

**(324)** (Found: C, 65.6; H, 7.61. $C_{23}H_{33}O_7$ requires C, 65.68; H, 7.68).

**(322)** $\delta_H$ ($CDCl_3$; 270MHz) 1.37, 1.54 (6H, 2s, C*Me*$_2$), 3.34 (1H, t, J 9.16, H-5, Ins), 3.49 (1H, dd, J 3.66, 8.80, H-3, Ins), 3.65 (1H, dd, J 6.96, 9.52, H-6, Ins), 3.79 (3H, s, Ins-O-C*H*$_2$PhOMe), 3.80 (3H, s, Ins-O-C*H*$_2$PhOMe), 3.84 (1H, t, J 8.80, H-4, Ins), 4.07 (1H, dd, J 5.68, 6.77, H-1, Ins), 4.18-4.33 (4H, m, Ins-O-C*H*$_2$CH=CH$_2$), 4.38 (1H, dd, J 3.84, 5.55, H-2, Ins), 4.70, 4.72 (2H, AB, J 10.44, Ins-O-C*H*$_2$PhOMe), 4.71, 4.77 (2H, AB, J 10.44, Ins-O-C*H*$_2$PhOMe), 5.15-5.36 (4H, m, Ins-O-CH$_2$CH=C*H*$_2$), 5.89-6.03 (2H, m, Ins-O-CH$_2$C*H*=CH$_2$), 6.85 (2H, d, J 8.61, Ins-O-CH$_2$*Ph*OMe), 6.86 (2H, d, J 8.79, Ins-O-CH$_2$*Ph*OMe), 7.27 (2H, d, J 8.61, Ins-O-CH$_2$*Ph*OMe), 7.28 (2H, d, J 8.61, Ins-O-CH$_2$*Ph*OMe).

$\delta_C$ ($CDCl_3$; 68MHz) 25.68, 27.60 (2q, C*Me*$_2$), 55.07 (q, Ins-O-CH$_2$PhO*Me*), 72.36, 72.78 74.79 (3t, Ins-O-*C*H$_2$CH=CH$_2$ and Ins-O-*C*H$_2$PhOMe), 74.79, 76.93, 78.91, 80.37, 81.73, 82.15 (6d, *C*H, *myo*-inositol ring carbons), 109.66 (s, Cq, *C*Me$_2$), 113.39, 113.58 (2d, Ins-O-CH$_2$*Ph*OMe), 116.63, 117.41 (2t, Ins-O-CH$_2$CH=*C*H$_2$), 129.25, 129.51, 129.67 (3d, Ins-O-CH$_2$*Ph*OMe), 130.64 (s, Cq, Ins-O-CH$_2$*Ph*OMe), 134.83, 135.05 (2d, Ins-O-CH$_2$*C*H=CH$_2$), 159.06 (s, Cq, Ins-O-CH$_2$*Ph*OMe).

m/z (-ve ion FAB) 693 [M + NBA, (10%)] 555 (33%) 525 (60%) 379 (20%) 137 [-O-CH$_2$PhOMe, (95%)] 121 [-CH$_2$PhOMe, (100%)].

**(324)** $\delta_H$ ($CDCl_3$; 270MHz) 1.37, 1.55 (6H, 2s, C*Me*$_2$), 2.72 (1H, d, J 2.02, $D_2O$ ex, Ins-OH), 3.43 (1H, ddd, J 2.02, 8.24, 8.24, $D_2O$ ex, dd, J 8.24, 8.24, H-5, Ins), 3.58 (2H, m, H-3 and H-6, Ins), 3.74 (1H, t, J 8.24, H-4, Ins), 3.79 (3H, s, Ins-O-CH$_2$PhO*Me*), 4.07 (1H, dd, J 5.68, 6.78, H-1, Ins), 4.17-4.36 (4H, m, Ins-O-C*H*$_2$CH=CH$_2$), 4.39 (1H, dd, J 3.85, 5.49, H-2, Ins), 4.70, 4.82 (2H, AB, J 10.80, Ins-O-C*H*$_2$PhOMe), 5.15-5.34 (4H, m, Ins-O-CH$_2$CH=C*H*$_2$), 5.87-6.03 (2H, m, Ins-O-CH$_2$C*H*=CH$_2$), 6.88 (2H, d, J 8.60, Ins-O-CH$_2$*Ph*OMe), 7.31 (2H, d, J 8.60, Ins-O-CH$_2$*Ph*OMe).

$\delta_C$ ($CDCl_3$; 68MHz) 25.62, 27.56 (2q, C*Me*$_2$), 55.07 (q, Ins-O-CH$_2$PhO*Me*), 72.07, 72.13, 74.30 (3t, Ins-O-*C*H$_2$CH=CH$_2$ and Ins-O-*C*H$_2$PhOMe), 73.43, 74.43, 77.00, 78.78, 79.98, 81.24 (6d, *C*H, *myo*-inositol ring carbons), 109.72 (s, Cq, *C*Me$_2$), 113.71 (d, Ins-O-CH$_2$*Ph*OMe), 117.09, 117.44 (2t, Ins-O-CH$_2$CH=*C*H$_2$), 128.48 (d, Ins-O-CH$_2$*Ph*OMe), 130.42 (s, Cq, Ins-O-CH$_2$*Ph*OMe), 134.63, 134.73 (2d, Ins-O-CH$_2$*C*H=CH$_2$), 159.12 (s, Cq, Ins-O-CH$_2$*Ph*OMe).

m/z (-ve ion FAB) 573 [M + NBA, (50%)] 419 [M - H, (28%)] 379 (38%) 299 [M - $CH_2$PhOMe, (100%)] 258 (38%) 137 (72%) 121 (80%).

**(323)** $\delta_H$ ($CDCl_3$; 270MHz) 1.37, 1.52 (6H, 2s, C*Me*$_2$), 2.68 (1H, d, J 1.47, $D_2O$ ex, Ins-OH), 3.23 (1H, t, J 9.16, H-5, Ins), 3.50 (1H, dd, J 3.84, 9.70, H-3, Ins), 3.50 (1H, dd, J 6.77, 9.16, H-6, Ins), 3.79 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.94 (1H, dt, J 1.46, 9.71, $D_2O$ ex, t, J 9.53, H-4, Ins), 4.10 (1H, dd, J 5.31, 6.78, H-1, Ins), 4.17-4.37 (4H, m, Ins-O-C*H*$_2$CH=$CH_2$), 4.41 (1H, dd, J 3.85, 5.31, H-2, Ins), 4.66, 4.81 (2H, AB, J 10.80, Ins-O-C*H*$_2$Ph), 5.15-5.34 (4H, m, Ins-O-$CH_2$CH=C*H*$_2$), 5.87-6.03 (2H, m, Ins-O-$CH_2$C*H*=$CH_2$), 6.86 (2H, d, J 8.79, Ins-O-$CH_2$*Ph*OMe), 7.31 (2H, d, J 8.80, Ins-O-$CH_2$*Ph*OMe).

$\delta_C$ ($CDCl_3$; 68MHz) 25.75, 27.75 (2q, C*Me*$_2$), 55.77 (q, Ins-O-$CH_2$PhO*Me*), 71.74, 72.62, 74.63 (3t, Ins-O-*C*$H_2$CH=$CH_2$ and Ins-O-*C*$H_2$PhOMe), 71.35, 73.75, 76.64, 79.14, 81.24, 82.09 (6d, *C*H, *myo*-inositol ring carbons), 109.79 (s, Cq, *C*Me$_2$), 113.78 (d, Ins-O-$CH_2$*Ph*OMe), 116.86, 117.86 (2t, Ins-O-$CH_2$CH=*C*$H_2$), 129.64 (d, Ins-O-$CH_2$*Ph*OMe), 130.45 (s, Cq, Ins-O-$CH_2$*Ph*OMe), 134.70, 134.92 (2d, Ins-O-$CH_2$*C*H=$CH_2$), 159.22 (s, Cq, Ins-O-$CH_2$*Ph*OMe).

m/z (-ve ion FAB) 573 [M + NBA, (100%)] 419 [M - H, (18%)] 379 (15%) 335 (20%) 299 [M - $CH_2$PhOMe, (100%)] 137 (72%).

### 6.5.3 DL-1,4-Di-*O*-allyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol (325)

A mixture of DL-1,4-di-*O*-allyl-2,3-*O*-isopropylidene-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(323)** (2.72g, 6.47mmol), and 1M HCl-methanol (90ml, 1:9), was heated at 50°C for 30min. The reaction mixture was cooled and TLC showed consumption of starting material to give a new spot $R_f$ = 0.20 (ether). Sodium hydrogen carbonate (5g) was added and the solvents were evaporated. The residue was partitioned between water (50ml) and dichloromethane (50ml), the organic layer was separated, dried over magnesium sulphate, filtered and evaporated to dryness. The remaining solid was recrystallised from ethyl acetate-hexane to give **(325)**. Yield, (1.97g, 80%).

m.p. 120-122°C (from ethyl acetate-hexane).
(Found: C, 62.9; H, 7.46. $C_{20}H_{28}O_7$ requires C, 63.12; H, 7.42).

$\delta_H$ ($CDCl_3$; 270MHz) 2.66, 2.85 (3H, 2s, $D_2O$ ex, Ins-OH), 3.32 (1H, dd, J 2.75, 9.53, H-3 or H-1, Ins), 3.44 (2H, dd, H-3 or H-1, and H-5 obscured, Ins), 3.59 (1H, t, J 9.34, H-4 or H-6, Ins), 3.72 (1H, t, J 9.34, H-4 or H-6, Ins), 3.80 (3H, s, Ins-O-$CH_2$PhO*Me*), 4.17-4.36 (5H, m, Ins-O-C*$H_2$*CH=$CH_2$, and H-2, Ins), 4.64, 4.86 (2H, AB, J 10.81, Ins-O-C*$H_2$*PhOMe), 5.16-5.35 (4H, m, Ins-O-$CH_2$CH=C*$H_2$*), 5.87-6.04 (2H, m, Ins-O-$CH_2$C*H*=$CH_2$), 6.88 (2H, d, J 8.61, Ins-O-$CH_2$*Ph*OMe), 7.31 (2H, d, J 8.61, Ins-O-$CH_2$*Ph*OMe).

$\delta_C$ ($CDCl_3$; 68MHz) 55.23 (q, Ins-O-$CH_2$PhO*Me*), 71.38, 73.82, 75.15 (3t, Ins-O-*C*$H_2$CH=$CH_2$ and Ins-O-*C*$H_2$PhOMe), 69.34, 71.51, 74.40, 79.59, 80.43, 80.60 (6d, *C*H, *myo*-inositol ring carbons), 113.87 (d, Ins-O-$CH_2$*Ph*OMe), 117.15, 117.57 (2t, Ins-O-$CH_2$CH=*C*$H_2$), 129.61 (d, Ins-O-$CH_2$*Ph*OMe), 130.64 (s, Cq, Ins-O-$CH_2$*Ph*OMe), 134.41, 135.05 (2d, Ins-O-$CH_2$*C*H=$CH_2$), 159.25 (s, Cq, Ins-O-$CH_2$*Ph*OMe).

m/z (-ve ion FAB) 533 [M + NBA, (65%)] 379 [M - H, (100%)] 303 (35%) 272 (20%) 182 (25%) 167 (30%).

### 6.5.4 DL-3,6-Di-*O*-allyl-1,2,5-tri-*O*-benzyl-4-*O*-*p*-methoxybenzyl-*myo*-inositol (326)

A mixture of DL-1,4-di-*O*-allyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(325)** (1.52g, 4mmol) and sodium hydride (0.864g, 36mmol) was stirred in dry DMF (40ml). Benzyl bromide (1.78ml, 15mmol), was added dropwise and the solution was stirred for a further 2h. TLC (ether-light petroleum, 1:1) showed one spot $R_f = 0.50$. The excess sodium hydride was destroyed with methanol (10ml) and the solvents were evaporated *in vacuo* to give a syrup. The syrup was partitioned between water (100ml) and ether then washed with 0.1M HCl (50ml), a saturated aqueous solution of sodium hydrogen carbonate (100ml) and water (100ml). The organic layer was dried over magnesium sulphate, filtered and the solvent evaporated. The remaining syrup was purified by flash chromatography to give the title compound **(326)**. Yield, (2.20g, 85%).

m.p. 53-54°C (from hexane).
(Found: C, 75.9; H, 7.19. $C_{41}H_{46}O_7$ requires C, 75.65; H, 7.13).

$\delta_H$ ($CDCl_3$; 270MHz) 3.22 (1H, dd, J 2.20, 9.71, H-3 or H-1, Ins), 3.29 (1H, dd, J 2.2, 9.89, H-3 or H-1, Ins), 3.38 (1H, t, J 9.34, H-5, Ins), 3.77 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.92 (1H, t, J 9.53, H-4 or H-6, Ins), 3.97 (1H, t, J 9.53, H-4 or H-6, Ins), 3.99 (1H, t, J 2.2, H-

2, Ins), 4.08-4.10 (2H, m, Ins-O-C$H_2$CH=$CH_2$), 4.27-4.43 (2H, m, Ins-O-C$H_2$CH=$CH_2$), 4.57-4.85 (8H, m, Ins-O-C$H_2$Ph and Ins-O-C$H_2$PhOMe), 5.11-5.34 (4H, m, Ins-O-$CH_2$CH=C$H_2$), 5.84-6.04 (2H, m, Ins-O-$CH_2$C*H*=$CH_2$), 6.83 (2H, d, J 8.61, Ins-O-$CH_2$*Ph*OMe), 7.22-7.42 (17H, m, Ins-O-$CH_2$*Ph* and Ins-O-$CH_2$*Ph*OMe).

$\delta_C$ ($CDCl_3$ 68MHz) 55.20 (q, Ins-O-$CH_2$PhO*Me*), 71.58, 72.82, 73.95, 74.53, 75.44, 75.83 (6t, Ins-O-*C*$H_2$CH=$CH_2$, Ins-O-*C*$H_2$Ph and Ins-O-*C*$H_2$PhOMe), 74.27, 80.60, 80.73, 81.25, 81.38, 83.58 (6d, *C*H, *myo*-inositol ring carbons), 113.68 (d, Ins-O-$CH_2$*Ph*OMe), 116.57 (t, Ins-O-$CH_2$CH=*C*$H_2$), 127.24, 127.44, 127.50, 127.73, 127.83, 128.05, 128.28, 129.74 (8d, Ins-O-$CH_2$*Ph* and Ins-O-$CH_2$*Ph*OMe), 131.07 (s, Cq, Ins-O-$CH_2$*Ph*OMe), 134.89, 135.38 (2d, Ins-O-$CH_2$*C*H=$CH_2$), 138.49, 138.91 (2s, Cq, Ins-O-$CH_2$*Ph*), 159.09 (s, Cq, Ins-O-$CH_2$*Ph*OMe).

m/z (-ve ion FAB) 803 [M + NBA, (100%)] 696 (35%) 665 (55%) 559 [M - benzyl, (25%)] 485 (40%) 440 (35%) 318 (68%) 287 (60%) 178 (30%) 124 (25%).

### 6.5.5 DL-3,6-Di-*O*-allyl-1,2,5-tri-*O*-benzyl-*myo*-inositol (327)

A mixture of DL-3,6-di-*O*-allyl-1,2,5-tri-*O*-benzyl-4-*O*-*p*-methoxybenzyl-*myo*-inositol **(326)** (1.70g, 2.61mmol) and DDQ (1.047g, 4.61mmol) in dichloromethane-water (20ml, 19:1) was stirred at room temperature for 1h. The precipitate was filtered and the organic layer was diluted with dichloromethane (150ml) and washed with 10% solution of sodium metabisulphite (3x100ml), a saturated solution of sodium hydrogen carbonate (100ml) and water (100ml). The organic layer was dried over magnesium sulphate, filtered and evaporated. The crude product was purified by flash chromatography to give **(327)** as a syrup, $R_f$ = 0.30 (ether-light petroleum, 1:1). Yield, (1.20g, 87%).

(Found: C, 74.7; H, 7.11. $C_{41}H_{46}O_7$ requires C, 74.67; H, 7.22 ).

$\delta_H$ ($CDCl_3$; 270MHz) 2.25 (1H, s, $D_2O$ ex, Ins-OH), 3.09 (1H, dd, J 2.20, 9.89, H-3 or H-1, Ins), 3.31 (1H, t, J 9.15, H-5, Ins), 3.32 (1H, dd, J 2.20, 9.76 H-3 or H-1, Ins), 3.90 (1H, t, J 9.52, H-4 or H-6, Ins), 3.93-4.10 (4H, Ins-O-C$H_2$CH=$CH_2$, H-2 and H-4, or H-6, Ins), 4.28-4.44 (2H, m, Ins-O-C$H_2$CH=$CH_2$), 4.62, 4.69 (2H, AB, J 11.91, Ins-O-C$H_2$Ph), 4.79, 4.83 (2H, AB, J 12.27, Ins-O-C$H_2$Ph), 4.85, 4.91 (2H, AB, J 11.17, Ins-O-C$H_2$Ph), 5.12-5.30 (4H, m, Ins-O-$CH_2$CH=C$H_2$), 5.80-6.05 (2H, m, Ins-O-$CH_2$C*H*=$CH_2$), 7.21-7.41 (15H, m, Ins-O-$CH_2$*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 71.06, 72.88, 73.91, 74.50, 75.28 (3t, Ins-O-*C*$H_2$CH=$CH_2$ and Ins-O-*C*$H_2$Ph), 72.55, 73.49, 79.69, 80.92, 81.15, 83.29 (6d, *C*H, *myo*-inositol ring carbons), 116.50 (t, Ins-O-$CH_2$CH=*C*$H_2$), 127.30, 127.46, 127.56, 127.72, 127.85, 128.08, 128.34 (7d, Ins-O-$CH_2$*Ph*), 134.47, 135.31 (2d, Ins-O-$CH_2$*C*H=$CH_2$), 138.40, 138.75, 138.82 (3s, Cq, Ins-O-$CH_2$*Ph*).

m/z (-ve ion FAB) 583 [M + NBA, (100%)] 576 (30%) 529 [M - H (8%)] 303 (35%) 489 (18%) 322 (12%) 273 (10%) 184 (10%).

### 6.5.6 DL-1,2,5-Tri-*O*-benzyl-*myo*-inositol (328)

A mixture of DL-3,6-di-*O*-allyl-1,2,5-tri-*O*-benzyl-*myo*-inositol **(327)** (0.8g, 1.78mmol), toluene-*p*-sulphonic acid (0.20g, 1mmol) and 10% palladium on activated charcoal (0.25g) in ethanol-water (35ml, 5:2), was heated under reflux for 5h. TLC (ether), showed a spot at $R_f = 0.30$ and debenzylation products at the baseline. The palladium on activated charcoal was filtered through celite to give a colourless solution, and the solvents were evaporated. The title compound **(328)** was purified by flash chromatography (dichloromethane-ethyl acetate, 1:1). Yield, (0.30g, 40%).

m.p. 161-162°C (from ethyl acetate-hexane).
(Found: C, 71.9; H, 6.71. $C_{27}H_{30}O_6$ requires C, 71.96; H, 6.49).

$\delta_H$ ($CDCl_3$; 270MHz) 1.65-2.65 (3H, very br, $D_2O$ ex, Ins-OH), 3.23 (1H, t, J 9.16, H-5, Ins), 3.30 (1H, dd, J 2.39, 9.71, H-1, Ins), 3.40 (1H, dd, J 2.75, 9.71, H-3, Ins), 3.82 (1H, t, J 9.52, H-4 or H-6, Ins), 4.06 (1H, t, J 2.57, H-2, Ins), 4.12 (1H, t, J 9.52, H-4 or H-6, Ins), 4.58-4.98 (6H, m, Ins-O-C*$H_2$*Ph), 7.25-7.40 (15H, m, Ins-O-$CH_2$*Ph*).

$\delta_C$ ($d_6$-DMSO; 68MHz) 71.51, 74.01 (2t, Ins-O-*C*$H_2$Ph), 72.16, 72.42, 72.84, 78.42, 80.43, 84.29 (6d, *C*H, *myo*-inositol ring carbons), 127.04, 127.14, 127.33, 127.56, 127.98, 128.08, 128.21 (7d, Ins-O-$CH_2$*Ph*), 139.20, 139.79, 139.92 (3s, Cq, Ins-O-$CH_2$*Ph*).

m/z (-ve ion FAB) 603 [M + NBA, (100%)] 379 [M - H, (85%)] 359 [M - benzyl (20%)] 335 (10%) 303 (20%) 151 (20%) 109 (5%).

### 6.5.7 DL-1,4-Di-*O*-allyl-5-*O*-benzyl-2,3-*O*-isopropylidene-6-*O*-*p*-methoxybenzyl-*myo*-inositol (329)

A mixture of DL-1,4-di-*O*-allyl-2,3-*O*-isopropylidene-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(324)** and sodium hydride (1.2g, 50mmol) was stirred in dry DMF (150ml) at room temperature. Benzyl bromide (2.37ml, 20mmol) was added dropwise which was then stirred for 2h after which TLC (ether-hexane, 1:1) showed a product, $R_f$ = 0.44. The excess sodium hydride was destroyed with methanol (10ml) and the solvents were evaporated *in vacuo*. The residue was partitioned between ether (300ml) and water (200ml) and the organic layer was washed with brine and water (200ml each). The organic layer was dried over magnesium sulphate and the solvent was evaporated. Flash chromatography (ether-hexane, 1:1) of the crude product provided the title compound **(329)** as a syrup. Yield, (7.1g, 93%).

(Found: C, 70.4; H, 7.64. $C_{30}H_{38}O_7$ requires C, 70.55; H, 7.51).

$\delta_H$ ($CDCl_3$; 270MHz) 1.38, 1.55 (6H, 2s, C*Me*$_2$), 3.36 (1H, t, J 9.16, H-5, Ins), 3.61 (1H, dd, J 3.84, 8.97, H-3, Ins), 3.67 (1H, dd, J 6.96, 9.52, H-4, Ins), 3.79 (3H, s, Ins-O-CH$_2$PhO*Me*), 3.86 (1H, t, J 8.79, H-6, Ins), 4.08 (1H, dd, J 5.68, 6.78, H-1, Ins), 4.20-4.37 (4H, m, Ins-O-C*H*$_2$CH=CH$_2$), 4.38 (1H, dd, J 3.85, 5.50, H-2, Ins), 4.68-4.83 (4H, Ins-O-C*H*$_2$PhOMe and Ins-O-C*H*$_2$Ph), 5.15-5.34 (4H, m, Ins-O-CH$_2$CH=C*H*$_2$), 5.88-6.02 (2H, m, Ins-O-CH$_2$C*H*=CH$_2$), 6.85 (2H, d, J 8.79, Ins-O-CH$_2$*Ph*OMe), 7.25-7.37 (7H, m, Ins-O-CH$_2$*Ph*OMe and Ins-O-CH$_2$*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 25.82, 27.75 (2q, C*Me*$_2$), 55.25 (q, Ins-O-CH$_2$PhO*Me*), 72.51, 72.93, 74.91, 75.91 (4t, Ins-O-CH$_2$CH=CH$_2$, Ins-O-*C*H$_2$Ph and Ins-O-*C*H$_2$PhOMe), 74.60, 77.08, 77.55, 79.08, 80.52, 82.26 (6d, *C*H, *myo*-inositol ring carbons), 109.84 (s, Cq, *C*Me$_2$), 113.80 (d, Ins-O-CH$_2$*Ph*OMe), 116.80, 117.50 (2t, Ins-O-CH$_2$CH=*C*H$_2$), 127.58, 128.02, 128.31, 129.68 (4d, Ins-O-CH$_2$*Ph* and Ins-O-CH$_2$*Ph*OMe), 130.83 (s, Cq, Ins-O-CH$_2$*Ph*OMe), 134.99, 135.21 (2d, Ins-O-CH$_2$*C*H=CH$_2$), 138.69 (s, Cq, Ins-O-CH$_2$*Ph*), 159.12 (s, Cq, Ins-O-CH$_2$*Ph*OMe).

m/z (+ve ion FAB) 419 [M - PMB, (1.2%)] 389 (3.3%) 121 (100%) 91 (45%).

### 6.5.8 DL-1,4-Di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol (330)

DL-1,4-Di-*O*-allyl-5-*O*-benzyl-2,3-*O*-isopropylidene-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(329)** (6.54g, 12.82mmol) was dissolved in methanol-1M HCl (100ml, 9:1), and the mixture was stirred at 50°C for 45min after which TLC (ether), showed one product, $R_f$ = 0.52. The reaction was cooled and 1M TEAB (20ml) was added. The solvents were evaporated to give a white solid. The solid was partitioned between dichloromethane (200ml) and water (200ml), and the organic layer was dried over magnesium sulphate, which was filtered and the solvent was evaporated to give a solid, **(330)**. Yield, (5.3g, 88%).

m.p. 87-88°C (from ether-hexane).
(Found: C, 68.7; H, 7.27. $C_{27}H_{34}O_7$ requires C, 68.9; H, 7.29).

$\delta_H$ ($CDCl_3$; 270MHz) 2.76 (2H, s, $D_2O$ ex, Ins-OH), 3.32 (1H, dd, J 2.93, 9.52, H-1, Ins), 3.38 (1H, t, J 9.34, H-5, Ins), 3.61 (1H, br d, J 9.52, $D_2O$ ex, dd, J 3.84, 9.52, H-3, Ins), 3.69 (1H, t, J 9.52, H-4 or H-6, Ins), 3.78 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.87 (1H, t, J 9.52, H-4 or H-6, Ins), 4.18-4.44 (5H, m, Ins-O-C*H*$_2$CH=$CH_2$ and H-2, Ins), 4.70-4.90 (4H, Ins-O-C*H*$_2$PhOMe and Ins-O-C*H*$_2$Ph), 5.14-5.35 (4H, m, Ins-O-$CH_2$CH=C*H*$_2$), 5.87-6.02 (2H, m, Ins-O-$CH_2$C*H*=$CH_2$), 6.83 (2H, d, J 8.79, Ins-O-$CH_2$*Ph*OMe), 7.24 (2H, d, J 8.43, Ins-O-$CH_2$*Ph*OMe), 7.27-7.35 (7H, m, Ins-O-$CH_2$*Ph*OMe and Ins-O-$CH_2$*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 55.19 (q, Ins-O-$CH_2$PhO*Me*), 71.71, 74.27 75.52 (3t, Ins-O-*C*H$_2$CH=$CH_2$, Ins-O-*C*H$_2$Ph and Ins-O-*C*H$_2$PhOMe), 69.31, 71.65, 79.79, 80.89, 81.20, 83.10 (6d, *C*H, *myo*-inositol ring carbons), 113.73 (d, Ins-O-$CH_2$*Ph*OMe), 117.09, 117.48 (2t, Ins-O-$CH_2$CH=*C*H$_2$), 127.50, 127.70, 128.30, 129.61 (4d, Ins-O-$CH_2$*Ph* and Ins-O-$CH_2$*Ph*OMe), 130.84 (s, Cq, Ins-O-$CH_2$*Ph*OMe), 134.56, 134.99 (2d, Ins-O-$CH_2$CH=$CH_2$), 138.63 (s, Cq, Ins-O-$CH_2$*Ph*), 159.16 (s, Cq, Ins-O-$CH_2$*Ph*OMe).

m/z (-ve ion FAB) 419 [M + NBA, (80%)] 469 [M - H, (100%)] 429 (15%) 273 (15%) 124 (10%).

### 6.5.9 DL-1,4-Di-*O*-allyl-2,3,5-tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol (326)

Benzyl bromide (0.83ml, 7mmol) was added to DL-1,4-di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(330)** (1.5g, 3.19mmol) and sodium hydride (0.48g, 20mmol) in dry DMF (20ml). The solution was stirred for 2h, after which TLC (ether-hexane, 1:1) showed a product $R_f = 0.50$. The excess sodium hydride was destroyed with methanol and the solvents were evaporated *in vacuo*. The residue was partitioned between dichloromethane and water (100ml of each), the organic layer was dried over magnesium sulphate, filtered and the solvent was evaporated to give the crude product. Flash chromatography, (ether-hexane, 1:1) gave the pure title compound **(326)**. Yield, (1.73g, 83%).

The NMR and mass spectrum data for compound **(326)** have been described previously in 6.5.5.

**6.5.10 DL-2,3,5-Tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-1,4-di-*O*-*cis*-prop-1-enyl-*myo*-inositol (331)**

A mixture of DL-1,4-di-*O*-allyl-2,3,5-tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(326)** (1.5g, 2.3mmol) and freshly sublimed potassium *t*-butoxide (3.66g, 30mmol) in dry DMSO (20ml) was kept at 50°C for 5h under an atmosphere of nitrogen. TLC (ether-hexane, 1:1) showed conversion of stating material $R_f = 0.50$ into a single product $R_f = 0.72$. The dark mixture was cooled and a saturated solution of potassium chloride (50ml) was added and the product was extracted with ether (4x50ml). The organic layer was dried over magnesium sulphate and evaporated to give a solid. The crude product was purified by flash chromatography (ether-hexane, 1:2) to give the title compound **(331)**. Yield, (1.2g, 80%).

m.p. 89-91°C (from hexane).
(Found: C, 75.6; H, 7.18. $C_{41}H_{46}O_7$ requires C, 75.65; H, 7.13).

$\delta_H$ ($CDCl_3$; 270MHz) 1.64 (3H, dd, J 1.65, 6.59, Ins-O-CH=CH-C*H*$_3$), 1.67 (3H, dd, J 1.47, 6.41, Ins-O-CH=CH-C*H*$_3$), 3.33 (1H, dd, J 2.38, 9.71, H-3 or H-1, Ins), 3.42 (1H, t, J 9.34, H-5, Ins), 3.51 (1H, dd, J 2.2, 9.89, H-3 or H-1, Ins), 3.76 (3H, s, Ins-O-CH$_2$PhO*Me*), 4.02 (1H, t, J 2.2, H-2, Ins), 4.03 (1H, t, J 9.89, H-4 or H-6, Ins), 4.17 (1H, t, J 9.34, H-4 or H-6, Ins), 4.36 (1H, dq, J 6.78, Ins-O-CH=C*H*-CH$_3$), 4.44 (1H, dq, J 6.78, Ins-O-CH=C*H*-CH$_3$), 4.53-4.83 (8H, m, Ins-O-C*H*$_2$Ph and Ins-O-C*H*$_2$PhOMe), 6.08 (1H, dd, J 1.65, 6.23, Ins-O-C*H*=CH-CH$_3$), 6.26 (1H, dd, J 1.65, 6.41, Ins-O-C*H*=CH-CH$_3$), 6.83 (2H, d, J 8.61, Ins-O-CH$_2$*Ph*OMe), 7.23-7.41 (17H, m, Ins-O-CH$_2$*Ph* and Ins-O-CH$_2$*Ph*OMe).

$\delta_C$ ($CDCl_3$ 68MHz) 9.27, 9.36 (2q, Ins-O-CH=CH-$CH_3$), 55.16 (q, Ins-O-$CH_2$PhO*Me*), 72.57, 74.45, 75.25, 75.69 (4t, Ins-O-$CH_2$Ph and Ins-O-$CH_2$PhOMe), 75.91, 78.75, 80.34, 82.53, 82.95, 84.36 (6d, *C*H, *myo*-inositol ring carbons), 98.10, 100.50 (2d, Ins-O-CH=*C*H-$CH_3$), 113.67 (d, Ins-O-$CH_2$*Ph*OMe), 127.34, 127.55, 127.78, 128.07, 128.17, 128.21, 128.26, 129.89 (8d, Ins-O-$CH_2$*Ph* and Ins-O-$CH_2$*Ph*OMe), 130.81 (s, Cq, Ins-O-$CH_2$*Ph*OMe), 138.27, 138.58 (2s, Cq, Ins-O-$CH_2$*Ph*), 145.70, 147.68 (2d, Ins-O-*C*H=CH-$CH_3$), 159.09 (s, Cq, Ins-O-$CH_2$*Ph*OMe).

m/z (-ve ion FAB) 803 [M + NBA, (100%)] 696 (35%) 665 (25%) 559 [M - benzyl (20%)] 485 (30%) 322 (35%) 273 (30%) 118 (24%).

### 6.5.11 DL-1,2,5-Tri-*O*-benzyl-*myo*-inositol (328)

DL-2,3,5-Tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-1,4-di-*O*-*cis*-prop-1-enyl-*myo*-inositol **(331)** (0.95g, 1.46mmol), was suspended in ethanol-1M HCl (60ml, 2:1). The mixture was heated at reflux temperature for 4h after which TLC (ether) showed a product $R_f = 0.30$. The solvents were evaporated and the solid was partitioned between water and dichloromethane (100ml each) and washed with sodium hydrogen carbonate (100ml) and water (100ml). The organic layer was then dried over magnesium sulphate and then evaporated. The title compound **(328)** was purified by flash chromatography (dichloromethane-ethyl acetate, 1:1). Yield, (0.55g, 84%).

The mass spectrum and NMR data have been described in (6.5.6).

### 6.5.12 DL-2,3,5-Tri-*O*-benzyl-1,4,6-tris[di(benzyloxyphospho)]-*myo*-inositol (333)

A mixture of bis(benzyloxy)diisopropylaminophosphine **(96)** (0.69g, 2mmol) and 1*H*-tetrazole (0.21g, 3mmol) in dry dichloromethane was stirred for 15min. DL-1,2,5-Tri-*O*-benzyl-*myo*-inositol **(328)** (0.2g, 0.44mmol) was then added and the reaction was stirred for a further 15min. The solution was cooled to -78°C and *t*-butylhydroperoxide (1ml, 7mmol) was added and the mixture was stirred for 30min. The reaction mixture was partitioned between dichloromethane and a 10% solution of sodium metabisulphite (100ml), the organic layer was washed with brine and water (100ml of each) and dried over magnesium sulphate. The remaining syrup was purified by flash chromatography (chloroform-acetone, 10:1), then ethyl acetate-pentane (2:1), to give **(333)** as a syrup. $R_f$ = 0.30 (chloroform-acetone, 10:1). Yield, (0.465g, 85%).

$\delta_H$ ($CDCl_3$; 400MHz) 3.41 (1H, dd, J 2.00, 9.76, H-3, Ins), 3.56 (1H, t, J 9.16, H-5, Ins), 4.21 (1H, dt, J 2.00, 9.46, H-1, Ins), 4.42 (1H, br s, H-2, Ins), 4.44-5.09 (18H, m, Ins-O-$CH_2$Ph, Ins-O-P(O)O-$CH_2$Ph, H-4 and H-6, Ins), 6.98-7.41 (45H, m, Ins-O-$CH_2$*Ph*, Ins-O-P(O)O-$CH_2$*Ph*).

$\delta_C$ ($CDCl_3$; 100MHz) 68.89, 69.21, 69.41, 69.77, 72.10, 73.53, 75.02 (7t, Ins-O-$CH_2$Ph and Ins-O-P(O)O-$CH_2$Ph), 74.57, 76.32, 77.49, 78.04, 78.78, 79.53 (6d, *C*H, *myo*-inositol ring carbons), 127.05, 127.47, 127.66, 127.89, 127.96, 128.05, 128.18, 128.47, 128.57 (9d, Ins-O-$CH_2$*Ph* and Ins-OP(O)O-$CH_2$*Ph*), 135.51, 135.77, 135.90, 137.10, 137.85, 137.98 (6s, Cq, Ins-O-$CH_2$*Ph* and Ins-OP(O)O-$CH_2$*Ph*).

$\delta_P$ ($CDCl_3$; 162MHz) -1.56, -1.86, -1.99 ($^{31}$P-$^{1}$H decoupled).

m/z (+ve ion FAB) 1231 [M + H, (80%)] 1141 (80%) 1051 (30%) 91 (100%).

### 6.5.13 DL-*myo*-Inositol 1,4,6-trisphosphate (334)

Ammonia was condensed into a three neck flask at -78°C. Small slithers of freshly cut sodium were added to the liquid ammonia until the colour remained blue. The ammonia was then distilled into a second flask and kept at -78°C. Sodium was then added once again until the solution remained blue. DL-2,3,5-Tri-*O*-benzyl-1,4,6-tris[di(benzyloxy phospho)]-*myo*-inositol **(333)** (0.1g, 81.3μmol) in dry dioxan (1ml), was then added to the sodium in liquid ammonia. The solution was stirred vigorously for 2min during which time the sodium in liquid ammonia remained blue. The reaction was quenched with methanol and the solvents were evaporated under a stream of nitrogen. The residue was dissolved in MilliQ water (250ml) and purified by ion exchange chromatography on Q-Sepharose Fast Flow, eluting with a gradient of TEAB buffer 0-1000mmol at pH 8.6. The triethylammonium salt of **(334)** eluted at *ca.* 600mmol buffer. Yield, (48.8μmol, 60%).

$\delta_H$ ($D_2O$; 270MHz) 3.61 (1H, t, J 9.16, H-5, Ins), 3.72 (1H, dd, J 2.38, 9.71, H-3, Ins), 4.07 (1H, t, J 9.52, H-1, Ins), 4.20 (1H, br s, H-2, Ins), 4.21 (1H, q, J 9.34, H-4, Ins), 4.33 (1H, q, J 9.34, H-6, Ins).

$\delta_P$ ($D_2O$; 109MHz) +1.01 (d, J 9.77, -CH-O-$PO_3^{2-}$), +1.44 (d, J 9.77, -CH-O-$PO_3^{2-}$), +2.02 (d, J 9.77, -CH-O-$PO_3^{2-}$).

m/z (-ve ion FAB) 419 [M - H, (70%)] 354 (25%) 291 (45%) 266 (30%) 201 (100%) 188 (30%) 113 (45%).

Accurate mass spectrum requires: $(M - H)^-$ = 418.9545. Found 418.9534.

### 6.5.14 DL-2,3,5-Tri-*O*-benzyl-1,4,6-tris[di(benzyloxyphosphorothio)]-*myo*-inositol (335)

A mixture of bis(benzyloxy)diisopropylaminophosphine **(96)** (1.035g, 3mmol), and 1*H*-tetrazole (0.35g, 5mmol) in dry dichloromethane (3ml) was stirred for 15min. DL-1,2,5-Tri-*O*-benzyl-*myo*-inositol **(328)** (0.20g, 0.44mmol) was added to the mixture which was then stirred for 10min. The solvent was evaporated, dry pyridine (1ml), dry DMF (2ml) and sulphur (0.192g, 6mmol) were added and the solution was stirred for 5min, after which sulphoxidation was completed as judged by $^{31}P$ NMR. The solvents were evaporated *in vacuo* at room temperature and the remaining syrup was partitioned between dichloromethane and 0.1M HCl (50ml each). The organic layer was then washed with 0.1M TEAB (50ml) and water (50ml). The dichloromethane layer was dried over magnesium sulphate and purified by flash chromatography (petroleum ether-ether, 2:1), $R_f$ = 0.52 and isolated as a syrup. Yield, (0.453g, 81%).

(Found: C, 65.0; H, 5.25. $C_{69}H_{69}O_{12}P_3S_3$ requires C, 64.78; H, 5.44).

$\delta_H$ ($CDCl_3$; 400MHz) 3.44 (1H, dd, J 1.47, 9.77, H-3, Ins), 3.64 (1H, t, J 10.07, H-5, Ins), 4.39-5.06 (20H, m, Ins-O-C$H_2$Ph, Ins-O-P(S)O-C$H_2$Ph, H-4 and H-6, Ins), 5.29 (1H, dt, J 9.46, 11.90, H-4, Ins), 5.38 (1H, dt, J 9.46, 12.21, H-6, Ins), 6.90-7.39 (45H, m, Ins-O-$CH_2$*Ph*, Ins-O-P(S)O-$CH_2$*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 69.44, 69.67, 69.86, 70.22, 71.89, 73.24, 74.73 (7t, Ins-O-*C*$H_2$Ph and Ins-O-P(S)O-*C*$H_2$Ph), 74.66, 76.88, 77.70, 78.07, 79.09, 79.51 (6d, *C*H, *myo*-inositol ring carbons), 126.69, 127.23, 127.42, 127.53, 127.70, 127.81, 128.05, 128.18, 128.26, 128.46 (10d, Ins-O-$CH_2$*Ph* and Ins-OP(S)O-$CH_2$*Ph*), 135.33, 135.58, 135.77, 135.82, 136.00, 137.25, 138.27, 138.37 (8s, Cq, Ins-O-$CH_2$*Ph* and Ins-OP(O)O-$CH_2$*Ph*).

$\delta_P$ ($CDCl_3$; 162MHz) +66.75 (dq, J 9.28, 9.52), +69.22 (dq, J 9.77, 10.50), +69.38 (dq, J 9.76, 10.74).

m/z (-ve ion FAB) 1186 [M - Bn, (10%)] 1096 (1%) 802 (1%) 293 (100%) 203 (10%) 95 (47%).

### 6.5.15 DL-*myo*-Inositol 1,4,6-trisphosphorothioate (336)

Ammonia was condensed into a three neck flask at -78°C. Pieces of freshly cut sodium were added to the liquid ammonia until the colour remained blue. The ammonia was then distilled into a second flask and kept at -78°C. Sodium was then added once again until, the solution remained blue. DL-2,3,5-Tri-*O*-benzyl-1,4,6-tris[di(benzyloxyphosphoro thio)]-*myo*-inositol **(335)** (0.1g, 78.2μmol) in dry dioxan (1ml), was then added to the sodium in liquid ammonia. The solution was stirred vigorously for 2min after which time the solution remained blue. The reaction was quenched with methanol (20ml) and the solvents were evaporated under a stream of nitrogen. The residue was dissolved in MilliQ water (250ml) and purified by ion exchange chromatography on Q-Sepharose Fast Flow, eluting with a gradient of TEAB buffer 0-1000mmol at pH 8.6. The glassy triethylammonium salt of **(336)** eluted at *ca.* 600mmol buffer. Yield, (31.28μmol, 40%).

$\delta_H$ ($D_2O$; 400MHz) 3.64 (1H, t, J 9.16, H-5, Ins), 3.74 (1H, d, J 9.46, H-3, Ins), 4.27 (1H, t, J 10.27, H-1, Ins), 4.34 (1H, br s, H-2, Ins), 4.44 (1H, q, J 9.76, H-4, Ins), 4.65 (1H, q, J 9.76, H-6, Ins).

$\delta_C$ ($D_2O$; 100MHz) 73.37, 73.76, 76.08, 77.74, 79.57, 80.28 (6d, *C*H, *myo*-inositol ring carbons).

$\delta_P$ ($D_2O$; 162MHz) +47.74 (d, J 10.20, -CH-O-*P*(S)$O_2^{2-}$), +48.49 (d, J 12.12, -CH-O-*P*(S)$O_2^{2-}$), +50.27 (d, J 11.29, -CH-O-*P*(S)$O_2^{2-}$).

m/z (-ve ion FAB) 466.3 [M - H, (100%)] 432 (10%) 371 (80%) 330 (5%) 113 (10%) 95 (17%).

Accurate mass spectrum requires: $(M - H)^-$ = 466.8860. Found 466.8871.

## 6.6 Synthesis of D- and L-*myo*-Inositol 1,4,6-Trisphosphate and D-*myo*-Inositol 1,4,6-Trisphosphorothioate

### 6.6.1 D-(337) And L-1-*O*-[*S*-(+)-*O*-acetylmandelyl]-3,6-di-*O*-allyl-5-*O*-benzyl-4-*O*-*p*-methoxybenzyl-*myo*-inositol (338)

A mixture of DL-1,4-di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(330)** (4.068g, 8.65mmol), *S*-(+)-*O*-acetylmandelic acid (1.75g, 9mmol) and DMAP (0.03g, 0.25mmol) in dry dichloromethane (10ml) was stirred at -20°C. A solution of DCC (1.96g, 9.5mmol), in dry dichloromethane (20ml) was added dropwise over 1.5h at -20°C and stirring continued overnight. TLC pentane-ethyl acetate (2:1), showed two products $R_f$ = 0.40 and $R_f$ = 0.28. The reaction mixture was filtered through celite which was washed thoroughly with dichloromethane (2x100ml). The solvent was evaporated to give a syrup which was purified by flash chromatography using pentane-ethyl acetate (2:1), to provide pure D-1-*O*-[*S*-(+)-*O*-acetylmandelyl]-3,6-di-*O*-allyl-5-*O*-benzyl-4-*O*-*p*-methoxybenzyl-*myo*-inositol **(337)** (2.04g, 36.5%) $R_f$ = 0.40 as a syrup, and L-1-*O*-[*S*-(+)-*O*-acetylmandelyl]-3,6-di-*O*-allyl-5-*O*-benzyl-4-*O*-*p*-methoxybenzyl-*myo*-inositol **(338)** (1.96g, 35%) as a solid.

**(338)** m.p. 103-105°C (from ethanol).
**(337)** $[\alpha]_D$ = -8° (c = 4.6 in $CH_2Cl_2$).
**(338)** $[\alpha]_D$ = +59° (c = 1 in $CH_2Cl_2$).
**(337)** (Found: C, 68.5; H, 6.55. $C_{37}H_{42}O_{10}$ requires C, 68.7; H, 6.55).
**(338)** (Found: C, 68.4; H, 6.50. $C_{37}H_{42}O_{10}$ requires C, 68.7; H, 6.55).

**(337)** $\delta_H$ ($CDCl_3$; 270MHz) 2.15 (3H, s, Ins-$O_2$C-CH-(O*Ac*)Ph), 2.65 (1H, s, $D_2O$ ex, Ins-OH), 3.31 (1H, dd, J 2.57, 9.71, H-3, Ins), 3.42 (1H, J 9.34, H-5, Ins), 3.71 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.85 (1H, t, J 9.34, H-4 or H-6, Ins), 3.94 (1H, t, J 9.53, H-4 or H-6, Ins), 4.08-4.29 (5H, m, Ins-O-C$H_2$CH=$CH_2$ and H-2, Ins), 4.77 (1H, dd, J 2.56, 10.26, H-1, Ins), 4.68-4.83 (4H, m, Ins-O-C$H_2$PhOMe, Ins-O-C$H_2$Ph), 5.07-5.28 (4H, m, Ins-O-$CH_2$CH=C$H_2$), 5.79-6.95 (2H, m, Ins-O-$CH_2$C*H*=$CH_2$), 5.97 (1H, s, Ins-$O_2$C-C*H*-(OAc)Ph), 6.80 (2H, d, J 8.61, Ins-O-$CH_2$*Ph*OMe), 7.21 (2H, d, J 8.61, Ins-O-$CH_2$*Ph*OMe), 7.24-7.52 (10H, m, Ins-O-$CH_2$*Ph*OMe, Ins-O-$CH_2$*Ph* and Ins-$O_2$C-C*H*-(OAc)*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 20.31 (q, Ins-$O_2$C-CH-(O*Ac*)Ph, -OC(O)*C*$H_3$), 54.88 (q, Ins-O-$CH_2$PhO*Me*), 71.53, 74.11, 75.22, 75.52 (4t, Ins-O-*C*$H_2$CH=$CH_2$, Ins-O-*C*$H_2$Ph and Ins-O-*C*$H_2$PhOMe), 67.37, 74.58, 77.86, 79.10, 80.34, 82.66, 82.85 (7d, *C*H, *myo*-inositol ring carbons and Ins-$O_2$C-*C*H-(OAc)Ph), 113.47 (d, Ins-O-$CH_2$*Ph*OMe), 116.52, 117.20 (2t, Ins-O-$CH_2$CH=*C*$H_2$), 127.16, 127.22, 127.48, 128.02, 128.55, 128.98, 129.33 (7d, Ins-O-$CH_2$*Ph*, Ins-O-$CH_2$*Ph*OMe and Ins-$O_2$C-CH-(OAc)*Ph*), 130.63, 133.24, 138.48 (3s, Cq, Ins-O-$CH_2$*Ph*OMe, Ins-O-$CH_2$*Ph* and Ins-$O_2$C-CH-(OAc)*Ph*), 134.18, 134.48 (2d, Ins-O-$CH_2$*C*H=$CH_2$), 158.91 (s, Cq, Ins-O-$CH_2$*Ph*OMe), 168.00, 170.40 (2s, Cq, Ins-$O_2$*C*-CH-(O*Ac*)Ph, -O*C*(O)$CH_3$).

m/z (-ve ion FAB) 799 [M + NBA, (28%)] 645 [M - H, (65%)] 469 (31%) 193 (50%) 175 (50%) 149 (100%).

**(338)** $\delta_H$ ($CDCl_3$; 270MHz) 2.18 (3H, s, Ins-$O_2$C-CH-(O*Ac*)Ph), 2.89 (1H, s, $D_2O$ ex, Ins-OH), 3.36 (1H, t, J 9.34, H-5, Ins), 3.36 (1H, dd, J 2.56, 9.52, H-3, Ins), 3.52 (1H, dd, J 5.5, 11.91, H-4 or H-6, Ins), 3.76 (3H, s, Ins-O-$CH_2$PhO*Me*), 3.77-4.23 (5H, m, Ins-O-*C*$H_2$CH=$CH_2$ and H-4 or H-6, Ins), 4.29 (1H, br s, $D_2O$ ex, J 2.57, H-2, Ins), 4.68-4.91 (7H, m, Ins-O-C*H*$_2$PhOMe, Ins-O-C*H*$_2$Ph, Ins-O-$CH_2$CH=C*H*$_2$ and H-1, Ins), 5.18-5.40 (3H, m, Ins-O-$CH_2$CH=C*H*$_2$ and Ins-O-$CH_2$C*H*=$CH_2$), 5.86-6.01 (1H, m, Ins-O-$CH_2$C*H*=$CH_2$), 5.97 (1H, s, Ins-$O_2$C-C*H*-(OAc)Ph), 6.82 (2H, d, J 8.79, Ins-O-$CH_2$*Ph*OMe), 7.27-7.35 (12H, m, Ins-O-$CH_2$*Ph*OMe, Ins-O-$CH_2$*Ph* and Ins-$O_2$C-C*H*-(OAc)*Ph*).

$\delta_C$ ($CDCl_3$; 68MHz) 20.54 (q, Ins-$O_2$C-CH-(O*Ac*)Ph, -OC(O)*C*$H_3$) 55.12 (q, Ins-O-$CH_2$PhO*Me*), 71.70, 73.87, 75.49 75.75 (4t, Ins-O-*C*$H_2$CH=$CH_2$, Ins-O-*C*$H_2$Ph and Ins-O-*C*$H_2$PhOMe), 67.61, 74.73, 74.92, 77.89, 79.29, 80.53, 82.85 (7d, *C*H, *myo*-inositol ring carbons and Ins-$O_2$C-*C*H-(OAc)Ph), 113.67 (d, Ins-O-$CH_2$*Ph*OMe), 116.26, 117.53 (2t, Ins-O-$CH_2$CH=*C*$H_2$), 127.42, 127.66, 127.97, 128.18, 128.81, 129.38, 129.56 (7d, Ins-O-$CH_2$*Ph*, Ins-O-$CH_2$*Ph*OMe and Ins-$O_2$C-CH-(OAc)*Ph*), 130.84, 133.14, 138.53 (3s, Cq, Ins-O-$CH_2$*Ph*OMe, Ins-O-$CH_2$*Ph* and Ins-$O_2$C-CH-(OAc)*Ph*), 134.28, 134.64 (2d, Ins-O-$CH_2$*C*H=$CH_2$), 159.11 (s, Cq, Ins-O-$CH_2$*Ph*OMe), 168.37, 170.48 (2s, Cq, Ins-$O_2$*C*-CH-(O*Ac*)Ph, -O*C*(O)$CH_3$).

m/z (-ve ion FAB) 799 [M + NBA, (68%)] 645 [M - H, (95%)] 469 (42%) 193 (55%) 175 (40%) 149 (100%).

### 6.6.2 L-1,4-Di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol (339)

A mixture of D-1-*O*-[*S*-(+)-*O*-acetylmandelyl]-3,6-di-*O*-allyl-5-*O*-benzyl-4-*O*-*p*-methoxy benzyl-*myo*-inositol **(337)**, (2.04g, 3.15mmol) and sodium hydroxide (0.8g, 20mmol) in methanol was heated at reflux temperature for 30min. The mixture was cooled and neutralised with carbon dioxide. The solid was diluted with water (50ml) and evaporated to dryness *in vacuo*. The product was extracted with dichloromethane (5x100ml) and the solvent was evaporated to give the product **(339)** $R_f = 0.52$ (ether). Yield, (1.34g, 90%).

m.p. 111-113°C (from ethyl acetate-hexane).
$[\alpha]_D = -51°$ (c = 1 in $CH_2Cl_2$).
(Found: C, 69.0; H, 7.32. $C_{27}H_{34}O_7$ requires C, 68.90; H, 5.29).

The mass spectrum and NMR data were the same as for the racemic mixture.

### 6.6.3 D-1,4-Di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol (340)

A mixture of L-1-*O*-[*S*-(+)-*O*-acetylmandelyl]-3,6-di-*O*-allyl-5-*O*-benzyl-4-*O*-*p*-methoxy benzyl-*myo*-inositol **(338)**, (1.96g, 3.03mmol) and sodium hydroxide (0.8g, 20mmol) in methanol was heated at reflux temperature for 30min. Work up was carried out in the same way as for **(339)** $R_f = 0.52$ (ether). Yield, (1.40g, 98%).

m.p. 111-113°C (from ethyl acetate-hexane).
$[\alpha]_D = +51°$ (c = 1 in $CH_2Cl_2$).
(Found: C, 68.8; H, 7.31. $C_{27}H_{34}O_7$ requires C, 68.90; H, 5.29).

The mass spectrum and NMR data were the same as for the racemic mixture.

### 6.6.4 L-1,4-Di-*O*-allyl-2.3,5-tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol (341)

A mixture of L-1,4-di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(339)** (1.70g, 3.6mmol) and sodium hydride (0.48g, 20mmol) in dry DMF (20ml) was stirred at room temperature. Benzyl bromide (1.07ml, 9mmol) was added to the mixture which was then stirred for 2h. The excess sodium hydride was destroyed with methanol (5ml) and the solvents were evaporated *in vacuo*. The residue was partitioned between ether and water (100ml of each), the organic layer was separated and dried over magnesium sulphate. The title compound **(341)** was purified by flash chromatography $R_f$ = 0.50 (ether-pentane, 1:1). Yield, (1.82g, 78%).

m.p. 72-73°C (from ethyl acetate-hexane).
$[\alpha]_D$ = -19° (c = 1 in $CH_2Cl_2$).
(Found: C, 75.3; H, 7.05. $C_{41}H_{46}O_7$ requires C, 75.6; H, 7.13).

The mass spectrum and NMR data were the same as for the racemic mixture.

### 6.6.5 D-1,4-Di-*O*-allyl-2.3,5-tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol (342)

A mixture of D-1,4-di-*O*-allyl-5-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(340)** (1.60g, 3.4mmol) and sodium hydride (0.48g, 20mmol) in dry DMF (20ml) was stirred at room temperature. Benzyl bromide (1.07ml, 9mmol) was added to the mixture which was then stirred for a further 2h. Work up and purification as for the L-enantiomer gave the pure title compound **(342)** $R_f$ = 0.50 (ether-pentane, 1:1). Yield, (2.077g, 94%).

m.p. 72-73°C (from ethyl acetate-hexane).
$[\alpha]_D$ = +19° (c = 1 in $CH_2Cl_2$).
(Found: C, 75.3; H, 7.96. $C_{41}H_{46}O_7$ requires C, 75.6; H, 7.13).

The mass spectrum and NMR data were the same as for the racemic mixture.

### 6.6.6 L-2,3,5-Tri-*O*-benzyl-*myo*-inositol (345)

A solution of L-1,4-di-*O*-allyl-2.3,5-tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(341)** (1.55g, 2.37mmol) and freshly sublimed potassium *t*-butoxide (3.66g, 30mmol) in anhydrous DMSO (30ml) was kept at 50°C for 5h, after which TLC (ether-hexane, 1:1) showed complete conversion of starting material, $R_f$ = 0.50, into a single product, $R_f$ = 0.72. The dark mixture was cooled water (50ml) was added and the product was extracted with ether (4x100ml). The organic layer was dried over magnesium sulphate and the solvent was evaporated to give an off-white solid **(343)**. This product was not isolated but treated with 1M aqueous HCl-ethanol (60ml, 1:2) for 3h, after which no starting material was left. The reaction was cooled and the solvents were evaporated *in vacuo*. The remaining solid was partitioned between dichloromethane and water (100ml of each) and the organic layer was dried over magnesium sulphate. The title compound

**(345)** was purified by flash chromatography (dichloromethane-ethyl acetate, 1:1), $R_f$ = 0.40 (ether). Yield, (0.71g, 67%).

m.p. 176-177°C (from ethyl acetate-hexane).
$[\alpha]_D$ = -34° (c = 1 in $CH_2Cl_2$).
(Found: C, 72.2; H, 6.72. $C_{27}H_{30}O_6$ requires C, 71.96; H, 6.49).

The mass spectrum and NMR data were the same as for the racemic mixture **(328)**.

### 6.6.7 D-2,3,5-Tri-*O*-benzyl-*myo*-inositol (346)

A solution of D-1,4-di-*O*-allyl-2,3,5-tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(342)** (1.2g, 1.84mmol) and freshly sublimed potassium *t*-butoxide (3.66g, 30mmol) in anhydrous DMSO (30ml) was kept at 50°C for 5h, after which TLC (ether-hexane, 1:1) showed complete conversion of starting material $R_f$ = 0.50 into a single product $R_f$ = 0.72. The product **(344)** was worked up, hydrolysed and purified in the same way for the L-enantiomer to give **(346)** with the same $R_f$ = 0.40 (ether) value. Yield, (0.589g, 71%).

m.p. 176-177°C (from ethyl acetate-hexane).
$[\alpha]_D$ = +34° (c = 1 in $CH_2Cl_2$).
(Found: C, 71.9; H, 6.75. $C_{27}H_{30}O_6$ requires C, 71.96; H, 6.49).

The mass spectrum and NMR data were the same as for the racemic mixture **(328)**.

### 6.6.8 D-2,3,5-Tri-*O*-benzyl-1,4,6-tris[di(benzyloxyphospho)]-*myo*-inositol (349)

A mixture of bis(benzyloxy)diisopropylaminophosphine **(96)** (1.036g, 4mmol) and 1*H*-tetrazole (0.42g, 6mmol) in dry dichloromethane (5ml) was stirred for 15min. D-2,3,5-Tri-*O*-benzyl-*myo*-inositol **(346)** (0.15g, 0.33mmol) was then added and the reaction was stirred for a further 15min. The solution was cooled to -78°C and *t*-butylhydroperoxide (1ml, 7mmol) was added and the mixture was stirred for 30min. The reaction mixture was partitioned between dichloromethane (100ml) and a 10% solution of sodium metabisulphite (100ml), the organic layer was washed with brine and water (100ml of each) dried over magnesium sulphate and evaporated. The remaining syrup was purified by flash chromatography (chloroform-acetone, 10:1), then ethyl acetate-pentane (2:1), to give **(349)** as a syrup. $R_f$ = 0.30 (chloroform-acetone, 10:1). Yield, (0.213g, 65%).

$[\alpha]_D = 0° \pm 1°$ (c = 3.6 in $CH_2Cl_2$).
(Found: C, 67.4; H, 5.42. $C_{69}H_{69}O_{15}P_3$ requires C, 67.31; H, 5.65).

The mass spectrum and NMR data were the same as for the racemic mixture.

### 6.6.9 L-2,3,5-Tri-*O*-benzyl-1,4,6-tris[di(benzyloxyphospho)]-*myo*-inositol (350)

A mixture of bis(benzyloxy)diisopropylaminophosphine **(96)** (0.69g, 2mmol) and 1*H*-tetrazole (0.42g, 6mmol) in dry dichloromethane (5ml) was stirred for 15min. L-2,3,5-Tri-*O*-benzyl-*myo*-inositol **(346)** (0.15g, 0.33mmol) was then added and the reaction was stirred for a further 15min. The solution was cooled to -78°C and *t*-butylhydroperoxide (1ml, 7mmol) was added and the mixture was stirred for 30min. The mixture was worked up in the same way as the D-enantiomer in order to obtain the product **(350)** as a syrup. $R_f$ = 0.30 (chloroform-acetone, 10:1). Yield, (0.236g, 72%).

$[\alpha]_D = 0° \pm 1°$ (c = 4.1 in $CH_2Cl_2$).
(Found: C, 67.2; H, 5.83. $C_{69}H_{69}O_{15}P_3$ requires C, 67.31; H, 5.65).

The mass spectrum and NMR data were the same as for the racemic mixture.

### 6.6.10 D-*myo*-Inositol 1,4,6-trisphosphate (199)

Ammonia was condensed into a three neck flask at -78°C. Freshly cut sodium was added to the liquid ammonia until the colour remained blue. The ammonia was then distilled into a second flask by gentle heating, and kept at -78°C. Freshly cut sodium was then added once again until the solution remained blue. D-2,3,5-Tri-*O*-benzyl-1,4,6-tris[di(benzyloxyphospho)]-*myo*-inositol **(349)** (0.11g, 89μmol) in dry dioxan (1ml), was then added to the sodium in liquid ammonia. The solution was stirred vigorously for 2min in which time the solution remained blue. The reaction was quenched with methanol (20ml) and the solvents were evaporated under a stream of nitrogen. The residue was dissolved in MilliQ water (250ml) and purified by ion exchange chromatography on Q-Sepharose Fast Flow, eluting with a gradient of TEAB buffer 0-1000mmol at pH 8.6. The triethylammonium salt of **(199)** eluted at *ca.* 700mmol buffer. Yield, (30.1μmol, 34%). (The NMR spectra were slightly different from the racemic mixture due to different pH values of the compound in the NMR tube).

$[\alpha]_D$ = -29.1° (c = 0.26 in TEAB, pH = 8.6).

$\delta_H$ ($D_2O$; 400MHz) 3.49 (1H, t, J 9.15, H-5, Ins), 3.58 (1H, dd, J 2.5, 9.77, H-3, Ins), 3.93 (1H, dt, J 2.5, 9.46, H-1, Ins), 4.07 (1H, br s, H-2, Ins), 4.07 (1H, q, J 9.16, H-4, Ins), 4.20 (1H, q, J 9.16, H-6, Ins).

$\delta_P$ ($D_2O$; 162MHz) +0.97 (d, J 8.3, -CH-O-$PO_3^{2-}$), +0.39 (d, J 8.79, -CH-O-$PO_3^{2-}$), -0.03 (d, J 9.52, -CH-O-$PO_3^{2-}$).

m/z (-ve ion FAB) 419 [M - H, (100%)] 339 (10%) 159 (7%) 97 (7%).

Accurate mass spectrum requires: $(M - H)^-$ = 418.9545. Found 418.9563.

### 6.6.11 L-*myo*-Inositol 1,4,6-trisphosphate (201)

The deprotection of L-2,3,5-tri-*O*-benzyl-1,4,6-tris[di(benzyloxyphospho)]-*myo*-inositol **(350)** (0.11g, 89μmol) was carried out in the same way as for the D-enantiomer **(349)**. Yield, (53.05μmol, 60%).

$[\alpha]_D$ = +25.0° (c = 0.88 in TEAB, pH = 8.6).

$\delta_H$ ($D_2O$; 270MHz) 3.58 (1H, t, J 9.16, H-5, Ins), 3.68 (1H, dd, J 2.5, 9.71, H-3, Ins), 4.04 (1H, dt, J 2.5, 9.46, H-1, Ins), 4.17 (1H, br s, H-2, Ins), 4.17 (1H, q, J 9.16, H-4, Ins), 4.29 (1H, q, J 9.16, H-6, Ins).

$\delta_P$ ($D_2O$; 162MHz) +2.11 (d, J 6.67, -CH-O-$PO_3^{2-}$), +1.44 (d, J 6.67, -CH-O-$PO_3^{2-}$), +1.04 (d, J 8.90, -CH-O-$PO_3^{2-}$).

m/z (-ve ion FAB) 419 [M - H, (100%)] 339 (10%) 159 (7%) 97 (7%).

Accurate mass spectrum requires: $(M - H)^-$ = 418.9545. Found 418.9523.

### 6.6.12 D-2,3,5-Tri-*O*-benzyl-1,4,6-tris[di(benzyloxyphorothio)]-*myo*-inositol (351)

A mixture of bis(benzyloxy)diisopropylaminophosphine **(96)** (1.035g, 3mmol), and 1*H*-tetrazole (0.35g, 5mmol) in dry dichloromethane (3ml) was stirred for 15min. D-2,3,5-Tri-*O*-benzyl-*myo*-inositol **(346)** (0.12g, 0.266mmol) was added to the mixture which was then stirred for 10min. The solvent was evaporated, dry pyridine (1ml), dry DMF (2ml) and sulphur (0.096g 3mmol) were added and the solution was stirred for 5min, after which sulphoxidation was completed as judged by $^{31}P$ NMR. The solvents were evaporated *in vacuo* at room temperature and the remaining syrup was partitioned between dichloromethane and 0.1M HCl (50ml each). The organic layer was then separated, washed with 0.1M TEAB (50ml) and water (50ml). The dichloromethane layer was dried over magnesium sulphate and purified by flash chromatography, (pentane-ethyl acetate, 3:1), $R_f = 0.52$ (ether-petroleum ether, 1:2) and isolated as a syrup. Yield, (0.28g, 82%).

$[\alpha]_D = 0° \pm 1°$ (c = 3.6 in $CH_2Cl_2$).
(Found: C, 65.1; H, 5.25. $C_{69}H_{69}O_{12}P_3S_3$ requires C, 64.78; H, 5.32).

The mass spectrum and NMR data were the same as for the racemic mixture **(335)**.

### 6.6.13 D-*myo*-Inositol 1,4,6-trisphosphorothioate (252)

The deprotection of L-2,3,5-tri-*O*-benzyl-1,4,6-tris[di(benzyloxyphosphorothio)]-*myo*-inositol **(351)** (0.11g, 86μmol) was carried out in the same way as for the enantiomer **(349)**. Yield, (31.44μmol, 36.5%).

$[\alpha]_D = -26.9°$ (c = 0.59 in TEAB, pH = 8.6).

$\delta_H$ ($D_2O$; 400MHz) 3.45 (1H, t, J 9.15, H-5, Ins), 3.56 (1H, dd, J 2.75, 9.77, H-3, Ins), 3.98 (1H, dt, J 2.44, 9.46, H-1, Ins), 4.24 (1H, q, J 9.46, H-4, Ins), 4.36 (1H, q, J 9.77, H-6, Ins), 4.41 (1H, br s, H-2, Ins).

$\delta_P$ ($D_2O$; 162MHz) +47.31 (d, J 9.92, -CH-O-$P(S)O_2^{2-}$), +48.24 (d, J 11.44, -CH-O-$P(S)O_2^{2-}$), +50.00 (d, J 11.45, -CH-O-$P(S)O_2^{2-}$).

m/z (-ve ion FAB) 467 [M - H, (100%)] 433 (15%) 334 (20%) 234 (20%) 95 (42%).

Accurate mass spectrum requires: $(M - H)^- = 466.8860$ Found 466.8871.

## 6.7 Establishing the Absolute Configuration of D-2,3,5-Tri-*O*-Benzyl-*myo*-Inositol

### 6.7.1 D-1,4-Di-*O*-allyl-2,3,5-tri-*O*-benzyl-*myo*-inositol (353)

D-1,4-Di-*O*-allyl-2,3,5-tri-*O*-benzyl-6-*O*-*p*-methoxybenzyl-*myo*-inositol **(342)** was suspended in 1M HCl-ethanol (60ml, 1:2). The mixture was heated at reflux temperature for 4h, after which TLC (ether-petroleum ether, 1:1), showed the appearance of a major product, $R_f = 0.40$. The reaction was cooled and the solvents were evaporated *in vacuo*. The remaining solid was taken up in dichloromethane (100ml) and washed with water (100ml). The organic layer was dried over magnesium sulphate filtered and the solvent was evaporated. The residue was purified by flash chromatography to give the title compound **(353)** as a syrup. Yield, (0.45g, 91%).

$[\alpha]_D = -3°$ (c = 9 in $CH_2Cl_2$).
(Found: C, 74.9; H, 7.41. $C_{33}H_{38}O_6$ requires C, 74.67; H, 7.22).

The mass spectrum and NMR data were the same as for the racemic mixture **(327)**.

### 6.7.2 D-1,4-Di-*O*-allyl-2,3,5,6-tetra-*O*-benzyl-*myo*-inositol (354)

A mixture of D-1,4-di-*O*-allyl-2,3,5-tri-*O*-benzyl-*myo*-inositol **(353)** (0.293, 0.55mmol) and sodium hydride (0.12g, 5mmol) was stirred in dry DMF (10ml) at room temperature. Benzyl bromide (0.12ml, 1.0mmol) was added and the reaction was stirred for a further 2h after which TLC (ether-pentane, 1:2) showed a product $R_f = 0.60$. The excess sodium hydride was destroyed with methanol and the solvents were evaporated *in vacuo*. The residue was partitioned between ether and water (50ml of each), the organic layer was then dried and evaporated to give a syrup. The residue was purified by flash chromatography (ether-pentane, 1:2) to give the title compound **(354)**. Yield, (0.31g, 90%).

m.p. 63-65°C (from pentane).
$[\alpha]_D = +18°$ (c = 1 in $CH_2Cl_2$).
(Found: C, 77.2; H, 7.15. $C_{40}H_{44}O_6$ requires C, 77.39; H, 7.14).

$\delta_H$ ($CDCl_3$; 400MHz) 3.23 (1H, dd, J 2.14, 9.77, H-3 or H-1, Ins), 3.30 (1H, dd, J 2.14, 10.07, H-3 or H-1, Ins), 3.40 (1H, t, J 9.15, H-5, Ins), 3.93 (1H, t, J 9.77, H-4 or H-6, Ins), 3.98 (1H, t, J 9.46, H-4 or H-6, Ins), 3.99 (1H, t, J 2.45, H-2, Ins), 4.04-4.12 (2H, m, Ins-O-$CH_2$CH=$CH_2$), 4.29-4.42 (2H, m, Ins-O-$CH_2$CH=$CH_2$), 4.60, 4.67 (2H, AB, J 10.8, Ins-O-$CH_2$Ph), 4.77-4.89 (6H, m, Ins-O-$CH_2$Ph), 5.12-5.31 (4H, m, Ins-O-$CH_2$CH=$CH_2$), 5.85-6.02 (2H, m, Ins-O-$CH_2$*CH*=$CH_2$), 7.23-7.41 (20H, m, Ins-O-$CH_2$*Ph*).

$\delta_C$ ($CDCl_3$ 100MHz) 71.63, 72.84, 73.97, 74.32, 75.84, 75.95 (6t, Ins-O-$CH_2$CH=$CH_2$, Ins-O-$CH_2$Ph), 74.59, 80.65, 80.77, 81.43, 81.58, 83.63 (6d, *C*H, *myo*-inositol ring carbons), 116.60, 116.66 (2t, Ins-O-$CH_2$CH=$CH_2$), 127.28, 127.48, 127.79, 127.94, 128.09, 128.31 (6d, Ins-O-$CH_2$*Ph*), 134.91, 135.42 (2d, Ins-O-$CH_2$*C*H=$CH_2$), 138.53, 138.86, 138.91, 138.95 (4s, Cq, Ins-O-$CH_2$*Ph*).

m/z (-ve ion FAB) 773 [M + NBA, (100%)] 666 (50%) 470 (30%) 322 (38%) 291 (30%) 140 (30%).

### 6.7.3 D-2,3,4,5-Tetra-*O*-benzyl-*myo*-inositol (355)

A mixture of D-1,4-di-*O*-allyl-2,3,5,6-tri-*O*-benzyl-*myo*-inositol **(354)** (0.26g, 1.78mmol), toluene-*p*-sulphonic acid (0.15g, 0.75mmol) and 10% palladium on activated charcoal (Fluka, 0.25g) was heated under reflux for 2h in ethanol-water (35ml, 6:1). TLC (ether), showed a new spot $R_f$ = 0.50 and debenzylated products at the baseline. The palladium on activated charcoal was filtered through celite to give a colourless solution, and the solvents were evaporated. The title compound **(355)** was purified by flash chromatography (dichloromethane-ethyl acetate, 1:1). Yield, (0.138g, 61%).

m.p. 103-105°C (from ether); (lit. [386] 105-107°C).
$[\alpha]_D$ = +4° (c = 1 in $CH_2Cl_2$); [lit. [386] $[\alpha]_D$ = +3.9° (c = 1 in $CHCl_3$)].
(Found: C, 75.6; H, 6.94. $C_{34}H_{36}O_6$ requires C, 75.53; H, 7.71).

$\delta_H$ ($CDCl_3$; 270MHz) 2.35 (1H, br s, $D_2O$ ex, Ins-OH), 2.61 (1H, br s, $D_2O$ ex, Ins-OH), 3.28 (1H, dd, J 2.20, 9.89, H-3, Ins), 3.38 (1H, t, J 9.15, H-5, Ins), 3.51 (1H, br d, J 9.53, $D_2O$ ex, dd, J 2.2, 9.53, H-1, Ins), 3.79 (1H, t, J 9.52, H-4, Ins), 4.05 (1H, t, J 2.20, H-2, Ins), 4.15 (1H, t, J 9.46, H-6, Ins), 4.56-4.93 (8H, m, Ins-O-$CH_2$Ph), 7.21-7.33 (20H, m, Ins-O-$CH_2$*Ph*).

$\delta_C$ ($CDCl_3$ 100MHz) 72.51, 74.63, 75.21, 75.46 (4t, Ins-O-*C*$H_2$Ph), 74.64, 73.25, 76.19, 80.39, 81.88, 83.28 (6d, *C*H, *myo*-inositol ring carbons), 127.65, 127.75, 127.78, 127.91, 128.04, 128.35, 128.43, 128.48, 128.56 (9d, Ins-O-$CH_2$*Ph*), 137.85, 138.60, 138.71 (3s, Cq, Ins-O-$CH_2$*Ph*).

m/z (-ve ion FAB) 692 [M + NBA, (100%)] 586 (40%) 539 (55%) 472 (15%) 322 (25%) 287 (20%).

## 6.8 Synthesis of Benzene 1,2,4-Trisphosphate

### 6.8.1 1,2,4-Tris-*O*-(diethoxyphospho)-benzene (357)

Benzene 1,2,4-triol **(355)** (0.252g, 2mmol) was suspended in dry dichloromethane (5ml) and stirred under a blanket of nitrogen. Dry *N,N*-diisopropylethylamine (2.1ml, 12mmol), was added to the suspension and the solution turned red in colour. The solution was then cooled to -78°C and diethoxychlorophosphine **(273)** (1.57ml, 9.0mmol) was added dropwise. The solution then turned pale yellow, which indicated the hydroxyl groups had been phosphitylated. The cooling was removed and water (2ml), was added to the solution which was stirred for 30min. *t*-Butylhydroperoxide (1ml, 7mmol, 70%), was added dropwise and the mixture was stirred for 15min at room temperature. TLC (ethyl acetate-ethanol, 9:1) showed a product $R_f$ = 0.34. The solution was diluted with dichloromethane (100ml), and washed with water (100ml), 10% sodium metabisulphite (100ml), 0.1M HCl (50ml), saturated sodium hydrogen carbonate solution (100ml) and water (100ml). The organic layer was dried over magnesium sulphate and evaporated to give the crude compound as an oil. Flash chromatography (ethyl acetate-ethanol, 9:1) gave the pure title compound **(357)** as a syrup. Yield, (0.79g, 76%).

(Found: C, 40.7; H, 6.42. $C_{18}H_{33}O_{12}P_3$ requires C, 40.45; H, 6.18).

$\delta_H$ ($CDCl_3$; 270MHz) 1.34-1.40 (18H, m, Bz-O-P(O)O-$CH_2C$*$H_3$*), 4.17-4.32 (12H, m, Bz-O-P(O)O-C*$H_2$*$CH_3$), 7.03-7.39 (3H, m, H-3, H-5, H-6, Bz).

$\delta_C$ ($CDCl_3$; 68MHz) 15.53, 15.63 (2q, Bz-O-P(O)O-$CH_2$*C*$H_3$), 64.43, 64.53, 64.64 (3t, Bz-O-P(O)O-*C*$H_2CH_3$), 113.29, 116.39, 121.43 (3d, *Bz*-O-P(O)O-$CH_2CH_3$), 138.22, 141.38, 146.91 (3s, Cq, *Bz*-O-P(O)O-$CH_2CH_3$).

$\delta_P$ ($CDCl_3$; 162MHz) -6.86 (dtt, J 7.83), -7.12 (dtt, J 7.83), -7.28 (dtt, J 7.83).

m/z (+ve ion FAB) 535 [M + H, (100%)] 507 (5%) 349 (12%) 269 (10%).

### 6.8.2 Benzene 1,2,4-trisphosphate (359)

1,2,4-Tris-*O*-(diethoxyphospho)-benzene, **(357)** (0.274g, 528µmol) was dissolved in dry dichloromethane. Bromotrimethylsilane (0.836ml, 6.33mmol) was added dropwise to the solution which was stirred for 16h. The solvents were evaporated and the residue was stirred with water 1ml. Final purification of **(359)** was by elution from Q-Sepharose Fast Flow using TEAB buffer with a linear gradient 0-1M. The title compound eluted between 0.2-0.5M buffer and after evaporation was obtained as its glassy triethylammonium salt. Yield, (456µmol, 86%).

$\delta_H$ ($D_2O$; 400MHz) 6.85 (1H, dd, J, 1.5, 8.85, H-5, Bz), 7.06 (1H, d, J 1.5, H-3, Bz) 7.18 (1H, d, J 8.85, H-6, Bz).

$\delta_P$ ($CDCl_3$; 162MHz) -3.61, (s) -3.92, (s) -4.28, (s).

m/z (-ve ion FAB) 364.9 [M - H, (100%)] 305 (27%) 267 (15%) 159 (10%).

Accurate mass spectrum requires: $(M - H)^-$ = 364.9228. Found 364.9238.

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